Hyg Acc # 2383

Digitized by the Internet Archive
in 2010 with funding from
University of Toronto

http://www.archive.org/details/journalofinfecti23infe

The
Journal of Infectious Diseases

Published by the John McCormick Institute for Infectious Diseases

EDITED BY

LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH

FRANK BILLINGS   F. G. NOVY
W. T. SEDGWICK   H. GIDEON WELLS

Library
School of Hygiene
University of Toronto

Volume 23
1918

Chicago, 1918

RC
110
J67
v.23
cop. 4

906004

Composed and Printed by
American Medical Association Press
Chicago, Illinois, U. S. A.

A BACTERIOLOGIC STUDY OF THE DIPHTHEROID ORGANISMS WITH SPECIAL REFERENCE TO HODGKIN'S DISEASE

Plates 1-3

Frederick Eberson

From the Department of Bacteriology, Columbia University, College of Physicians and Surgeons, New York City

I. NOMENCLATURE AND CLASSIFICATION OF THE DIPHTHEROIDS

Introduction

Within the past few years the literature dealing with organisms of the diphtheria group have received many new contributions. It seems that most human and animal diseases of hitherto unknown etiology have been ascribed to some sort of diphtheroid organism. Whether or not these results will be confirmed has little bearing on the problem which confronts the systematic bacteriologist who finds himself hard put to it in attempting to classify the great number of organisms which for no reason other than a slight resemblance to the diphtheria bacteria have been thrown haphazardly into a group of "unknowns"—the diphtheroids.

Since Loeffler's discovery of the diphtheria organism, so many so-called "pseudodiphtheria" bacteria have been mentioned in the literature that it seems necessary at this time to survey the field and come to an understanding as to what constitutes a diphtheroid.

In a recent paper on the "Nomenclature of the Coccaceae," Buchanan has pointed out the chaotic state of bacterial nomenclature. That the naming of species, genera and higher groups needs careful study and revision is a foregone conclusion. A great need is felt for such an analysis in the groups of bacteria which possess common characteristics. Such a group is the diphtheroid group.

First, it is necessary to define a "diphtheroid." The term "pseudodiphtheria" has always seemed unsatisfactory because the organisms so-called differ in so many respects from the true Klebs-Loeffler organism as to warrant the dropping of the term from the literature except in conditions to be suggested herewith. A review of the extensive

Received for publication Feb. 6, 1918.

work done in this group reveals the fact that there are bacteria which resemble the true diphtheria organism morphologically and culturally, but which differ in one important respect, namely, that of virulence as tested on guinea-pigs. The true Klebs-Loeffler has been shown to ferment dextrose, maltose and dextrin with acid production and to possess definite toxic properties on injection, resulting in the death of the animal in 3-4 days with a characteristic inflammation and congestion of the suprarenal glands. To those bacteria which lack this virulence but which otherwise conform to the character of true diphtheria bacteria, should be assigned the species designation "pseudodiphtheria." The generic name given to the group by Lehmann and Leumann is Corynebacterium and should be retained for the organisms of this group. Certain characters ought to be accepted as properly belonging to the species before we can adequately define what we mean by a diphtheroid. These may be enumerated as follows: Morphological resemblance to diphtheria organism, gram-positive, nonmotile, absence of spores, presence or absence of metachromatic granules, no gas-production in carbohydrate mediums. The fermentation of sugars may or may not occur. This property gives us a basis for classification which will be taken up later. A careful study of the different species which have been described shows that the characters enumerated ought rightly to be accepted as a basis for differentiating the diphtheroids from other bacteria which have been improperly classed with them. With this end in view the different species are taken up individually and examined from the standpoint of validity and priority. The suitability of the name is considered from the viewpoint of sufficient description, and in the light of more comprehensive studies which have given us suggestions for proper nomenclature. When the species name implies etiology which is unknown, doubtful or unconfirmed, that part of it is modified to suit the case.

HISTORICAL REVIEW OF DIPHTHEROIDS AND EMENDATIONS

B. pseudodiphtheriticum (Loeffler) Migula

The description is given as follows: Gram-positive, no polar bodies, nonmotile. Grows luxuriantly on blood serum with a glistening layer. In broth clouding is produced. Loeffler described a pseudodiphtheria organism in his first paper on the etiology of diphtheria. He said the organism resembled diphtheria but was avirulent for guinea-pigs; that it was smaller than the true diphtheria and showed a marked absence of club-shaped forms. In 1888, v. Hofmann published an account of avirulent diphtheria-like organisms isolated from the oral cavity in 26 of 45 normal persons. He called these organisms pseudodiphtheria bacilli and described them as follows: Clouding produced in broth, no acid production, grow at room temperature, no spores.

The rightful name for this species is Corynebacterium pseudodiphtheriticum (Hofmann-Wellenhof). It is questionable, however, if we can retain this name for all diphtheroids which are morphologically and culturally like the one described by v. Hofmann. Recent studies show that there are different species of saprophytic diphtheroids which conform to the type characters, yet are not found in the nose or throat. At the close of this communication we shall describe a few such strains which have been isolated from widely varying sources.

B. PSEUDODIPHTHERITICUS ALCALIFACIENS

B. PSEUDODIPHTHERITICUS ACIDUM FACIENS

These names were applied by Kurth to diphtheroid organisms which he found to be avirulent, and were capable of producing, respectively, a weakly alkaline and an acid reaction in sugar broth. The alcalifaciens species conforms to the following description: No granules, short forms on serum with white growth, abundant growth in bouillon with clouding, no acid produced in sugar broth.

This description, although meager, suggests that Kurth was undoubtedly dealing with the type Hofmanni. B. alcalifaciens should lapse into synonymy with this species. As for the B. pseud. acidum faciens a new species is hardly justified in view of the fact that avirulent diphtheroids differ in their fermentative properties in various sugars. Moreover the description is incomplete. Apart from the interest which attaches to the first attempt at classification from the fermentation standpoint, these two species have no particular value.

B. XEROSIS (NEISSER AND KUSCHBERT) MIGULA

Club-shaped, plump, and longer septate forms. On blood serum terminal swellings never noted. Gram-positive, polar bodies sometimes visible, no growth below 25 C., no clouding in bouillon, but granular sediment is formed. Distinguished from pseudodiphtheria by its ability to grow only above 25 C.

One point needs slight modification in this description of B. xerosis, namely, that of temperature requirement. On certain mediums, such as ascitic dextrose agar we have noted growth of 12 strains of diphtheroids which were isolated from the eye. These organisms complied with the description given for B. xerosis as regards morphology and fermentative properties in different carbohydrate broths. Some of the strains developed very slowly at temperatures ranging from 18-25 C., showing delicate growth on slant mediums after 4 days. Eisenberg states that B. xerosis grows only at body temperature.

B. SEPTATUM GELPKE

Nonmotile, gram-positive, club-shaped forms on serum. Young cultures show polar staining, no spores. Grows on serum with a delicate yellowish dry appearance. In bouillon no clouding occurs but a granular flaky deposit is visible. Does not grow at temperatures below 28 C. Is to be distinguished from other organisms of the group by this property. Associated with conjunctival inflammation—"schwellungskatarrh."

A comparison of the 2 organisms just described makes it appear questionable that we are dealing with a distinct species in B. septatum. This organism which appears to be identical with B. xerosis is, like the latter, not necessarily associated with a conjunctivitis for B. xerosis has been isolated frequently from normal eyes. Zinsser is of the opinion that the B. xerosis is a harmless parasite which may occur more often in the slightly inflamed than in the normal conjunctiva. The term septatum is not especially characteristic for diph-

theroids associated with normal and diseased eyes. Large striped or septate bacteria have been found in the nose and in the blood during the course of personal study on this group of organisms.

B. NODOSUS PARVUS (LUSTGARTEN-MANNABERG)

Club-shaped organism, disposed at angles or parallel nonmotile, spores absent, slow growth at 37 C. on agar with white appearance, nonpathogenic, facultative. Found in normal human urethra. Migula has changed the name of this bacterium somewhat and calls it B. nodosum. Similar organisms have been isolated from the urine. In one case we have found this organism associated with a gonorrheal urethritis and in another instance in normal urine. Marked differences have been noted in the acid-forming properties of these 2 strains. One gives rise to abundant acid in several sugars and the other fails to attack any of them. They are gram-positive. Hine mentions 11 strains of diphtheroids which he isolated from the urogenital tract. They appeared as large clubbed forms, markedly segmented and exhibited granules, often of large size. They fermented dextrose, saccharose, maltose and dextrin.

It is suggested that the name Corynebact. nodosum (Migula) be given to these organisms and that B. nodosum parvum lapse into synonymy.

B. ENDOCARDITIS GRISEUS (WEICHSELBAUM)

Flügge classifies this species under the head of pseudodiphtheria. It is a short rod, morphologically resembling B. xerosis. Grows well at room temperature, facultative, gram-positive, irregular staining property, is actively motile, glistening growth on mediums, with grayish-white appearance. In streak cultures a light brown or reddish-gray growth is observed. Pathogenic for guinea-pigs and white mice. Rabbits, when injected, show endocarditis. Found on the heart valves in a fatal case of recurrent ulcerative endocarditis. According to the suggested definition for diphtheroid this bacillus should be ruled out. The property of motility has not been observed among members of the diphtheria group.

B. ERYTHEMATIS

Demme, in 1887, described an organism which caused erythema with death in guinea-pigs. It is a delicate rod, disposed in smaller or larger groups, nonmotile, forms spores, grows best at 37 C. but is slow in development, facultative, gram-positive. Flügge has put this bacterium in the diphtheroid group. Apart from the diphtheroid picture which might be seen in a microscopic field because of the configuration in groups and by virtue of the bulging forms due to spores, there is no apparent reason for classifying this species as a diphtheroid. Spore-formation rules the organism out of the group.

B. RENALIS BOVIS (ENDERLEN) MIGULA

Rods with thickened ends, nonmotile, gram-positive. In broth, a granular precipitate is formed, the mediums remaining clear. Obligate aerobe. Does not grow at room temperature. A more adequate description is given by Ernst. He isolated the strain from cases of pyelonephritis in cattle. The organism grows poorly as compared with the diphtheria bacterium. Babes-Ernst granules appear much later. It is club-shaped, lancetlike or cylindrical. No growth takes place at room temperature. No acid produced in dextrose and glycerin, no spores, facultative aerobe, nonmotile, gram-positive. Pathogenicity appears doubtful. Ernst was unable to demonstrate virulence for guinea-pigs or rab-

bits by means of intraperitoneal or intrapulmonary inoculations. Concludes that the organism is not etiologic for pyelonephritis, since he could not recover the organism from the lesions or reproduce the disease.

Grips named an organism which he isolated from swine Bacillus pyogenes suis. Künnemann described a similar organism from cattle under the name B. pyogenes bovis. Glage combined the two under the single specific name B. pyogenes. Glage and Prieve, in their studies have shown that the organisms are related to the influenza bacillus and are members of the group of hemoglobinophilic organisms. The name C. pyogenes (Glage) is perhaps most suitable for this organism.

Among human beings, pus cavities and suppurating wounds have been found to harbor diphtheroid organisms. Whether or not these possess pyogenic properties is speculative. It may be of interest to note that three strains isolated by the writer have been obtained from an anal pus pocket in a patient whose kidney underwent suppuration and complete necrosis. These organisms have been injected subcutaneously and intraperitoneally into guinea-pigs with negative results. Diphtheroids found in sinuses following abscess formation are not rare. It is very likely that these organisms are skin inhabitants.

B. STRIATUS FLAVUS (V. BESSER)

Eisenberg's description: Found in normal nasal mucus. Short thick rods, sometimes curved, with striped or barred appearance. On agar white, thick growth with yellow pigment. Grows rapidly at room temperature, no spores.

B. STRIATUS ALBUS (V. BESSER)

Same source as B. striatus flavus. Appearance the same. Growth on agar is white and glistening, no spores. Flügge mentions B. striatus albus only. Chester has named the organism B. striatum.

That these striped diphtheroids occur in the normal nasal mucus has been shown by several investigators. Undoubtedly v. Besser was dealing with but one type of organism which is capable of producing pigment varying from white to yellow. In a comprehensive study made by Morse this was found to be the case. Our own studies have confirmed these findings. Morse has given this species diphtheroid the name B. flavidus with the following diagnosis: Thick forms with clear cut bars. Large and irregular granules visible. Heavy growth on serum with pigment varying in color from white to yellow. Ferments dextrose and maltose but not saccharose. B. flavidus as a species designation is invalid since a prior description had already been given by v. Besser and later amended by Chester to conform with binomial system of nomenclature. The emendation by Morse is recognized but the species should be written. Coryn. striatum (Chester) nov. comb.

B. BORDONI—UFFREDUZZI (EISENBERG)

This is known as B. epidermidis and has been described as Bizzozeros Leptothrix epidermidis. It is an avirulent spore-former growing best at 15-20 C. Since this organism does not conform to the genus requirements, it should not be classed with the diphtheroids.

B. CLAVATUS

Flügge, in 1894, gave this name to a club-shaped bacillus which he isolated from milk heated to 100 C. for one hour. It is a long bacillus, motile, forms spores. Swollen at ends, anaerobic, liquefies gelatin.

This same species is met with in an earlier work on dysentery by Kruse and Pasquale. They isolated from the heart blood and mesenteric fluid in cases of Egyptian dysentery an organism which they call "B. clavatus." It is not described in any way as to warrant its recognition as a species. The characters enumerated by Flügge make it invalid as a type of corynebacteria.

SPORE-FORMING DIPHTHEROID OF DE SIMONI

Isolated from nasal secretion in case of ozena. Said to form spores only when grown in milk or on potato. Facultative anaerobe. Grows luxuriantly in dextrose, lactose and maltose broth with slight acid formation.

The author was undoubtedly misled by the appearance of an impure culture. The illustration given in his article shows a complete transformation from a typical diphtheroid of slender and unevenly staining aspect to a blunt spore-bearing rod in older cultures. This is highly improbable, yet were it absolutely authentic, one should not be inclined to class this species with the diphtheria group for reasons outlined previously.

B. DIPHTHEROIDES

Described by Klein. Isolated from purulent secretion of udder of cow. Organism fails to grow below 25 C. Growth on agar slow and sparse, aerobic. On serum grows best at 37 C., producing white colonies. It is oval or spherical in form with a deeply staining center. Club-shaped forms present, gram-positive. Produces abscess in guinea-pigs after subcutaneous or intraperitoneal inoculation. The organism dies within a week when grown on artificial mediums.

This diphtheroid, according to the few cultural characters given, is in all likelihood identical with C. pyogenes (Glage). The description is too meager to warrant retaining it as a distinct species. The fact that the organism died so readily on artificial mediums indicates that it resembled the hemoglobinophilic type of which Glage's is an example.

B. VARIABILIS LYMPHAE VACCINALIS

This is the first of several descriptions of a species associated with the vaccine pustules of calves. Nakanishi, in 1900, reported on an organism which he isolated from a vaccine pustule. It is club-shaped, coccoid, segmented or branched. Stains poorly with Gram, facultative anaerobe, nonmotile. Central granule noted when stained with warm fuschin. Growth on serum is abundant. Pale yellow or orange-yellow pigment produced. Poor growth on gelatin. Spore-formation is questionable. The name of this species is not a binomial and therefore may be discarded. A somewhat later and more adequate description by Levy and Fickler is invalid, however, for the same reason. Their organisms were found to be identical with that of Nakanishi and possessed pyogenic properties for guinea-pigs, mice and rabbits. The name given to the species by these authors is Corynebact. lymphae vaccinalis. It is definitely gram-positive. In 1904 this species is met with as Coryneb. pyogenes, so described by Lewandowsky who published a paper almost simultaneously with Galli-Valerio. The last named author found his species to be identical with Nakanishi's and that of Levy and Fickler. Galli-Valerio proposes the name Coryneb. vaccinae (Galli-Valerio). Lewandowsky's designation is a binomial and valid. Galli-Valerio gave no reasons for rejecting a prior description and was apparently unaware of Lewandowsky's work which appeared at the same

time. The species is best described perhaps as Coryneb. vaccinae (Galli-Valerio) because the name C. pyogenes had already been used by Glage (q. v.) for an organism isolated from cattle.

B. coryzae segmentosus Cautley; B. diphtheroides citreus; B. diphtheroides brevis; B. maculatus; B. diphtheroides liquefaciens; B. auris; B. ceruminis.

These seven strains were described by Graham Smith. He isolated the first 5 from the nose and mouth, and the last 2 from the ear. B. coryzae segmentosus was first cited by Cautley in a local English government report. The organism is characterized as follows: Nonmotile, gram-positive, polar bodies visible, white growth in gelatin. In broth, clear with sediment. Slight acid produced in dextrose bouillon. Avirulent.

The species' name is objectionable from the etiologic standpoint and violates the rule for binomial nomenclature. This organism has been isolated from normal nasal mucus on numerous occasions and is not necessarily associated with coryza. It differs from C. striatum in its slight acid-producing power in dextrose, its slow growth and small polar bodies. That this species may be obtained from the eye as well as from the nose has been noted by the writer. In these instances it is to be differentiated from C. xerosis by the absence of acidity when grown in maltose and saccharose broths. As a rule C. xerosis ferments these last named sugars (Morse). It is suggested that the name Coryneb. segmentosum nom. nov. be substituted for the invalid B. coryzae segmentosus Cautley. B. diphtheroides citreus corresponds to Coryn. striatum (Chester). It produces yellow pigment. The name given by Graham Smith is not a binomial and disregards priority. It should lapse into synonymy with Coryn. striatum (Chester) Eberson.

B. DIPHTHEROIDES BREVIS

From pus of oral abscess. Growth on agar, white and slimy. In gelatin, white and dry, clouding in bouillon, granular sediment. Highly acid in sugar broth. This species will be discussed later in connection with diphtheroids isolated from the ear. This organism B. maculatus is meagerly described as an acid-producer. Avirulent. It is impossible to tell wherein this organism agrees with or differs from the main types which have been established in recent studies and for that reason can hardly be looked on as a distinct species until more complete biometrical study is made for strains from a similar source.

B. DIPHTHEROIDES LIQUEFACIENS

On serum growth is light yellow. Very long bacillus, somewhat curved, with polar bodies. Slightly motile; gram-positive; on agar, growth is thick and moist with light yellow pigment. Gelatin is liquefied in 3 days. In bouillon, slight clouding with granular sediment. In sugar broth reaction is weakly alkaline or neutral. Avirulent. A search through the literature indicates that gelatin liquefying properties have not been associated with diphtheroids, except in this one instance. In 61 strains studied by the writer, but one was found to liquefy gelatin. This species will be described under isolations from ascitic fluids and Graham Smith's organism will be discussed and emended.

B. AURIS

From aural pus of scarlet fever patient. On serum is similar to that of Klebs-Loeffler organism, but slower in growth. Segmented, nonmotile, gram-positive, polar bodies visible. On agar, gray colonies; on gelatin, small trans-

lucent colonies which become white in three days. Bouillon is clouded slightly and sediment produced. Visible acid produced in sugar broth.

B. CERUMINIS

From normal ear. Long, thin, curved organism. Gram-positive, polar bodies. Bouillon remains clear with sediment. Neutral or alkaline reaction in sugar broth. Avirulent.

DIPHTHEROIDS FROM THE EAR

Diphtheroids of the ear have been made the subject of a very valuable paper by Alice Hamilton. In a study of over 50 cases of otitis media and scarlet fever she succeeded in differentiating 2 groups of organisms.

GROUP 1.—Includes diphtheroids with the following characters: Short rods, gram-positive (weakly staining). Abundant growth on agar. Ferment dextrose and saccharose but not maltose or dextrin. Nonpathogenic.

GROUP 2.—Long, barred or granular, often clubbed. Gram-positive, scanty growth on agar. Ferment dextrose and maltose, but not saccharose. Often virulent for guinea-pigs.

The pathogenicity of these organisms is altogether different from that of the diphtheria bacterium inasmuch as diphtheria antitoxin did not protect against them. Hamilton's studies showed also that the organisms isolated from otitis undoubtedly have some bearing on the disease since vaccine treatment had a marked effect on opsonic determinations. If we compare this type (Group 2) with the B. auris described by Graham Smith it appears that we have the same organism. Unfortunately the work of the last named author does not mention virulence tests and the strains were too few in number to merit comparison. We are safe, however, in recognizing that the diphtheroids found in the ear are not of the same type and that in cases of otitis media and post-scarlatinal infections another species, not necessarily of etiologic significance, seems to predominate. The name Coryn. auris (Graham Smith) is proposed.

B. CERUMINIS

This organism has been observed also by Dr. Dwyer, of this laboratory. This organism fails to ferment any of the sugars and differs from C. Hofmanni in morphology as well as its behavior in broth which remains clear, with sediment. The name should be Coryn. ceruminis (Graham Smith) comb. nov.

There remains for consideration the diphtheroids of Hamilton's Group 1. These organisms differ from C. hoagii (Morse) only in the absence of a salmon-pink pigment. In all likelihood these strains conform to this type. Besides these ear diphtheroids, there seems to be a variety which is characterized by vigorous fermentative properties in a number of sugars. Two such strains have been isolated by the writer. A description follows: Gram-positive, small rods, abundant, white glistening growth on agar, no spores. Form large amounts of acid in dextrose, lactose, saccharose, maltose, mannite, and dextrin, but do not split raffinose. Acidity titrations with N/20 NaCH after 48 hours' incubation were as follows:

	Dextrose	Lactose	Saccharose	Maltose	Mannite	Dextrin
Strain 1	3.2	3.5	3.9	4.0	2.8	2.3
Strain 2	5.0	3.6	4.4	4.7	2.6	2.0

These strains were obtained from cases of otitis media. This species is similar to the diphtheroides brevis described by Graham Smith. It is suggested that a

binomial be used for the species with the emendation noted above, namely, Coryn. acidum sp. nov.

B. PSEUDODIPHTHERITICUS GAZOGENES

This is the only gas-forming diphtheroid met with in the literature. Jacobson has described it as follows: Isolated from stools of an infant. Is a vigorous gas former, club-shaped with thickened ends. "One of the cultures resembled streptobacilli. This character did not persist in the transplants." Gram-positive and "in cultures a few days old gram-negative organisms were observed." No spores. Good growth in gelatin, after 5-6 days. Litmus milk is reddened in 24 hours. Nonpathogenic. From the description given by the author it does not seem likely that we have to deal with one organism. Several things point to the impurity of the culture. Isolation from feces, as was here the case, makes such contamination all the more probable. According to the definition of a diphtheroid, this organism which is here described is hardly to be reckoned as one of the genus Corynebacterium.

DIPHTHEROIDS AND ACNE VULGARIS

Unna, in 1891, repeatedly found in lesions of acne vulgaris an organism which he concluded was the cause of the disease. He did not isolate the organism in pure culture, but gave it the name "Flaschenbacillus" and described it as follows: Unevenly staining rod having clubbed ends and showing ellipsoid forms. In 1894, Hodara, in a bacteriologic study of acne, reported the presence of two types of bacteria in acne lesions. One type is that of Unna and the other Hodara considers as another variety which is similar in appearance but shows varying morphology. For this species he used the name "Flaschen-Kugel-Bacillus" ("Bacillus á Flacons-Ballons").

Neither of these papers goes into any detail concerning the organisms and are of little, if any, value for classification purposes. The first accurate report on these diphtheroids is given by Sabouraud about 3 years later. Sabouraud studied the bacterial flora of the sebaceous plugs which can be squeezed out of the skin. He found an organism which he claimed to be the true cause of the disease according to his definition, and which he described as follows: Punctiform bacillus, almost coccoid, gram-positive, less than 1 mikron in length. Stains readily with anilin dyes. Requires highly acid mediums for growth. Produces intense clouding in broth. Sabouraud named the organism "bacille de séborrhée grasse."

It was not until 1901, when Gilchrist made a thorough study on the bacterial flora of the skin, that an adequate description is found for the bacteria which were mentioned by Unna and his followers. Gilchrist's description points to the identity of his organism with that of his predecessors. It is gram-positive, stains unevenly and very small. It is anaerobic, although many strains were found to grow aerobically after continued cultivation on artificial culture mediums. Surface smears made with the material from pustules failed to show growth whereas stab cultures and heavily inoculated unbroken pustules always showed growth. The organism is named B. acnes by the author. We are still in doubt as to the cause of acne and for this reason B. acnes as a species designation may be criticized. Fleming claims to have obtained beneficial results by the use of autogenous vaccines prepared from similar organisms which he isolated from cases of acne. As Sabouraud pointed out these diphtheroids are found in sebaceous plugs and recent observations of numerous workers as well as of our own, show that these bacteria, anaerobic in character, can be isolated

very readily from "blackheads" without the use of special mediums. It is believed by some that "blackheads" are to be looked on as a stage of acne. In order to adhere to the rules of priority we should designate this diphtheroid as follows; Coryn. acnes (Gilchrist) comb. nov. Cultural studies with this organism were made by Südmerson and Thompson in 1910. They confirmed Sabouraud's findings as to the suitability of highly acid mediums for cultivating C. acnes. One part horse serum to 3 parts of 3% nutrient agar (4.0) is recommended as a culture medium for isolation. Ordinary glucose agar may be used just as well, however. Fermentation studies show that the organism ferments dextrose, saccharose (slight), maltose, mannite and inulin. Raffinose is fermented by 1 strain and not by another. The authors suggest raffinose as a means for differentiating 2 species. Several strains studied by the writer formed no acid in raffinose and in dextrin.

Virulence tests were made on guinea-pigs and mice. Only the latter succumbed to an injection of 4 loopfuls of a 5 day culture. On necropsy the animals showed a greatly enlarged spleen, enlarged and congested mesenteric glands, enlarged, soft and anemic kidney, congested suprarenals and an enlargement and congestion of the axillary and inguinal glands.

B. PSEUDOTUBERCULOSIS OVIS PREISZ (NOCARD)

This organism was originally isolated by Preisz and Guinard from necrotic areas in the kidney of sheep. Later, Preisz described the organism more fully. It is gram-positive, nonmotile and club-shaped. On serum it produces a heavy moist growth with golden-yellow pigment. Toxin production is attributed to the organism and is fatal for guinea-pigs, rabbits, goats and sheep. In guinea-pigs, intraperitoneal inoculation gives rise to an orchitis. In larger animals there is produced a pseudotuberculosis which can be differentiated from true tuberculosis by histologic study of the pseudotubercles which do not show the characteristic giant cells. In a recent paper by Hall and Stone this organism was found to produce suppurative processes throughout the lymphatics in guinea-pigs. The toxin which is elaborated by the bacterium resembles but is not identical with that of diphtheria, yet is partially neutralized by diphtheria antitoxin. The authors found that the organism was hemolytic in blood agar which did not contain an excess of fermentable carbohydrate. Dextrose and maltose were fermented, but glycerol was not attacked, thus differing from C. striatum which ferments glycerin and like the B. pseudotuberculosis ovis Preisz produces a yellow pigment. Three strains of this organism (horse, sheep, calf) which were sent to the Museum of Natural History by the writer were tested for acid production along with the other diphtheroids. The strain from the calf produced more acid in dextrose and dextrin than either of the other two. The detailed results of the tests were as follows:

	N/20 acid			
	Dextrose	Lactose	Saccharose	Dextrin
(Horse)	1.8	0.6	0.3	0.7
(Sheep)	0.9	0.3		
(Calf)	2.4	0.4	0.3	1.7

Coryn pseudotuberculosis (Preisz) comb. nov. is perhaps the correct designation.

B. HOAGII

Morse described this type in 1912 as representing one of the groups which she established as the result of a biometrical study of 295 strains. The characters may be summarized as follows: Medium-sized bacterium showing solid

barred and wedged forms with abundant but small and imperfect granules. On serum it produces a very heavy, confluent, glistening growth with a characteristic salmon-pink color. Ferments dextrose and saccharose, but not maltose. The organism was described by Hoag, but not named by him. The species should be written Corynebacterium hoagi (Morse) comb. nov.

CORYNEBACTERIUM HODGKINII

This name is applied to an organism said to be the cause of a glandular disease about which pathologists and bacteriologists are still at odds. In recent years Bunting and Yates, Rosenow, and Billings have been strong adherents to the belief that Hodgkin's disease is caused by a diphtheroid organism which they have cultivated from glandular material obtained from such patients. Organisms similar to those of Bunting and Yates have been isolated from Hodgkin's cases by Rhea and Falconer recently, and according to the earlier work of Fraenkel and Much, and de Negri and Mieremet have been found in similar cases. Fraenkel and Much thought that the organism was related to the tubercle bacillus since it was found by them to be resistant to antiformin. This character has been shown to be insignificant by de Negri and Mieremet who observed the contrary. This test was made on 60 strains of diphtheroids by the writer and in no case was an antiformin-resistant type observed. The first description of this diphtheroid is given by Fraenkel and Much as follows: Granular, antiformin-resistant, gram-positive, nonacid fast. Avirulent for guinea-pigs. de Negri and Mieremet add several cultural characters: Plump short rod, granular, polar staining. Grows best at 32 C. and prefers aerobic conditions and alkaline reaction of mediums. Abundant growth on Loeffler's serum, with slimy consistency. Bordet medium of blood-glycerin, potato-extract and agar gives abundant growth in 24 hours. According to Bunting and Yates, gelatin is not liquefied and broth is not clouded, but sediment is formed. In this paper it is not proposed to discuss the etiology of Hodgkin's disease, but in reviewing the work of others and from personal studies, it seems that we are not yet ready to ascribe to these diphtheroids the great importance which has been given to them. That organisms similar to these can be isolated from normal individuals as well as from a variety of diseased conditions has been well shown by Bloomfield who made a study of the bacterial flora of lymphatic glands. He found avirulent organisms which were correlated with saprophytes on the body surface in cases of Hodgkin's disease, lymphosarcoma, carcinoma. Another group of organisms seemed to possess a relation to oxygen supply. These were of frequent occurrence. They are short, pleomorphic rods, gram-positive, nonacid fast, no polar bodies and exhibit clubbed forms. Bloomfield found these in cases of lymphosarcoma, arthritis, carcinoma and Hodgkin's disease. Virulence tests on rabbits, guinea-pigs and mice were negative. He concludes that definitely diseased glands yield a greater number of successful cultures than do normal glands. Saprophytic organisms are filtered out by the glands and become a permanent flora of the same. None of the isolated strains appears to be the etiologic factor in specific diseases. Another study by Fox has thrown further light on the question of secondary invasion. Fox found that no one bacterial variety with definite morphologic and cultural characters has been isolated from cases of Hodgkin's disease. That these diphtheroids may be found in enlarged glands in other conditions is mentioned by Fox and has been observed by the writer who has isolated organisms morphologically and culturally identical with the so-called C. Hodgkinii, from an hypertrophied tonsil and from lymph nodes and other sources. For the sake of comparison the fol-

lowing table is given so as to show the lack of uniformity in Hodgkin's strains which our study has so far illustrated.

A glance at Table 1 shows that the strains isolated from Hodgkin's cases not only differ among themselves, but are not to be distinguished from organisms obtained from widely varying sources. The study of Hodgkin's disease is to be the subject of another report. Until the etiology of Hodgkin's disease is more definitely ascertained, it is suggested that C. Hodgkinii should not be retained as a valid species inasmuch as the two C. Hodgkinii (Rosenow) strains are widely different in cultural characters. In view of the fact that other organisms which are in no way related to this disease or any adenopathy resembling it, are culturally and morphologically identical with this organism, the name Hodgkinii serves no purpose and leads to confusion.

TABLE 1

SHOWING THE LACK OF UNIFORMITY IN ACID PRODUCTION IN HODGKIN'S STRAINS

Culture	Source	N/20 Acid Produced in					
		Dextrose	Lactose	Saccharose	Maltose	Mannite	Dextrin
1	Hodgkin's (Bunting)	0.2	—	—	0.3	—	—
2	Hodgkin's (Bunting)	0.7	—	—	1.1	—	0.2
3	Lymphocytic leukemia	0.3	—	—	0.5	—	—
4	Acute pseudoleukemia	0.2	2.5	—	0.5	—	0.3
5	Hodgkin's (Bunting)	0.2	—	—	0.3	—	—
6	Blood (Bunting)	0.2	—	—	0.3	—	—
8	C. Hodgkinii (Rosenow)	2.8	—	1.9	2.7	—	0.3
10	Blood (Eberson)	—	—	—	0.2	—	—
11	Tonsil (Eberson)	0.2	—	—	0.4	—	—
12	Anal pus pocket (Eberson)	—	—	—	0.3	—	—
13	C. Hodgkinii (Rosenow)	0.1	—	—	0.3	—	—
15	Bone marrow (Eberson)	0.2	—	—	0.2	—	—
28	Hodgkin's (Rosenow)	0.9	—	—	1.7	—	—
34	Hodgkin's (Rosenow)	2.0	—	1.7	2.9	—	0.3
38	Hodgkin's (Rosenow)	1.9	—	—	2.2	—	0.3
44	Lymph node (Eberson)	—	0.1	—	0.3	—	—
49	Hodgkin's (Eberson)	0.1	—	—	0.4	—	—
51	Hodgkin's (Eberson)	1.5	—	1.7	1.7	—	0.3

DISTRIBUTION OF DIPHTHEROIDS

At this point it seems convenient to tabulate the diphtheroids which have been found in glands and tissues and arrange the several species which have not been classified heretofore.

DIPHTHEROIDS IN GLANDS AND TISSUES

The following signs indicate acid production: ±, 0.1 to 0.5 N/20 NaOH required for neutralization; +, 0.6 to 1.0 N/20 NaOH required for neutralization; ++, 1.1 to 2.5 N/20 NaOH required for neutralization; +++, 2.6 to 4.0 N/20 NaOH required for neutralization; —, no acid; alk, weakly alkaline.

Sugar broths were prepared as follows: 1% by weight of each of the carbohydrates were added to meat infusion broth prepared according to standard methods. The carbohydrates used were Merck's C. P. lactose, saccharose, maltose, mannite and raffinose and Kahlbaum's dextrose and dextrin. Difco peptone was used in preparation of broth. To the mediums (sterilized in Arnold for 20 minutes on 3 successive days and immediately cooled) was

added sterile ascitic fluid in the proportions of 1 in 10. The mediums were then incubated at 37 C. for 24 hours and 1 day at room temperature to test for sterility. For inoculation, 48-hour cultures of the diphtheroids grown on ascitic dextrose agar (0.5% dextrose) were used. Titrations were made in the cold after 48 hours' incubation, using N/20 NaOH. Five c c of the broth were used for titration. Instead of adding 45 c c of water and a few drops of phenolphthalein to each 5 c c of material, it was found more convenient to add phenolphthalein to a large volume of water and then to make this exactly neutral by addition of a small amount of N/1 NaOH, as was necessary for the water used in the tests. In this way, errors due to variations in reaction of dis tilled water may be avoided. This method and the procedure followed in the preparation of mediums were adhered to in all titrations.

TABLE 2

THE ACID-PRODUCING PROPERTIES OF DIPHTHEROIDS FOUND IN GLANDS AND TISSUES

Culture	Source	Dextrose	Lactose	Saccharose	Maltose	Mannite	Dextrin	Raffinose
B.* 1	Hodgkin's disease	±	—	—	±	—	—	—
B. 2	Hodgkin's disease	+	—	—	+	—	±	±
B. 3	Axillary gland, lymphocytic leukemia	±	—	—	±	—	—	—
B. 4	Cutaneous tumor, acute pseudoleukemia	±	++	—	+	—	±	++
B. 5	Hodgkin's disease	±	—	—	±	—	—	alk
B. 6	Hodgkin's disease	±	—	—	±	—	—	—
R. 8	C. Hodgkinii	+++	—	++	+++	—	±	alk.
8t	Hypertrophied tonsil	—	—	—	—	—	—	—
10	Blood	±	—	—	±	—	—	—
11	Hypertrophied tonsil	±	—	—	±	—	—	—
R. 13	C. Hodgkinii	±	—	—	±	—	—	—
13t	Hypertrophied tonsil	—	—	—	—	—	—	—
14t	Tonsil	—	—	—	—	—	—	—
15	Bone marrow, leukemia	±	—	—	±	—	—	alk.
27t	Hypertrophied tonsil	—	—	—	—	—	—	—
R. 28	Hodgkin's disease	+	—	—	++	—	—	—
29	Hypertrophied tonsil	—	—	—	—	—	—	—
R. 34	Hodgkin's disease	++	—	++	+++	—	±	alk.
35	Hypertrophied tonsil	±	—	—	±	—	±	—
R. 38	Hodgkin's disease	++	—	—	++	—	±	alk.
44	Lymph node	—	±	—	±	—	—	—
47	Brain, meningitis	+++	+++	—	++	—	—	—
49	Hodgkin's disease	±	—	—	±	—	—	—
51	Hodgkin's disease	++	—	++	++	—	±	—

* B. = Bunting strains; R. = Rosenow strains.

Diphtheroids isolated from glands and certain tissues fall into several groups: (1) Those which ferment dextrose and maltose slightly; (2) those which ferment dextrose, saccharose, maltose vigorously and dextrin slightly; (3) those which ferment dextrose and maltose vigorously; (4) those which ferment dextrose and maltose vigorously and dextrin slightly; (5) those which ferment lactose, maltose and raffinose vigorously, and dextrin and dextrose slightly; (6) those which ferment lactose and maltose slightly; (7) those which ferment none of the sugars; and (8) those which ferment dextrose, lactose and maltose vigorously. That the range of fermentative powers varies widely is

seen at a glance. Before any systematic classification is attempted many more strains must be isolated from lymph glands and tissues. A few new strains will be described here, however.

The diphtheroids which were isolated from tonsils, of which 224 were studied, varied in morphology from very small rods to large club-shaped forms. They were all gram-positive, nonmotile, nonacid fast, nonspore forming, aerobic, and failed to produce gas in carbohydrate mediums. Four were hemoglobinophilic. A few of those strains possessed hemolytic properties. One produced an orange pigment when grown on ascitic dextrose agar slants. This property was observed on ordinary agar as well. Diphtheroids with orange pigment had not been described in the literature at this time, and it is suggested that this organism be called C. aurantiacum. A similar species was obtained from a Hodgkin's gland. The pigment production was studied from the standpoint of oxygen and temperature requirements. At room temperature (18-25 C.) under aerobic and anaerobic conditions, pigment was produced abundantly in from 24-48 hours. At 37 C. under aerobic conditions, the orange color was abundant in the same interval of time, whereas under anaerobic conditions, a very slight coloration was observed after 10-14 days. The organism is gram-positive, nonmotile, does not form spores, is not acid fast and is facultative. On ascitic dextrose agar, it grows abundantly with an orange tinge after 24 hours. It is a small bacterium, coccoid in appearance. No polar bodies or granules seen when stained with the Neisser stain. It grows well in dextrose, lactose, saccharose, maltose, mannite, dextrin and raffinose broth and produces acid in dextrose and maltose. At room temperature abundant growth takes place. Gelatin is not liquefied.

Hemolysis of rabbit corpuscles by diphtheroids is not usually attributed to any but the true Klebs-Loeffler bacterium from severe cases (Schwoner). The organisms isolated from the tonsils were in all cases but one, culturally unlike the diphtheria organism and in this one instance proved to be an avirulent diphtheroid, for which the name C. pseudodiphtheriae sp. nov. is proposed. This organism grows on plain agar, is morphologically like the Klebs-Loeffler bacillus. It shows no growth anaerobically after 7 days and is apparently strictly aerobic. Some of the strains from tonsils required blood for growth and died off rapidly unless transferred frequently. These organisms resembled B. influenzae and were hemoglobinophilic.

Three strains were found which fermented dextrose, saccharose, and maltose vigorously and formed slight acid in dextrin. Raffinose remained neutral or faintly alkaline. These diphtheroids were isolated from glands in patients with Hodgkin's disease. In sugar broths, growth was abundant with even clouding. At room temperature, on ascitic agar abundant growth occurs. At 37 C. on the same medium growth is abundant with a heavy creamy consistency and brownish tinge. The organisms, small, ovoid or coccoid, stain readily with anilin dyes, are gram-positive, facultative, nonmotile, do not form spores, are not acid-fast. On rabbit blood-agar plates, brown pigment is formed along the streak growths. On Loeffler's serum, abundant white, glistening growth takes place. One strain, on first isolation, from the gland on blood plates, exhibited a remarkable likeness to B. tuberculosis in character of growth. The colonies were dry and heaped up and friable. This peculiar property did not persist in later transplants. Antiformin treatment showed these diphtheroids to be nonresistant. The name C. glandulae sp. nov. is suggested for this species.

While this study was under way, a paper by Torrey appeared dealing with bacteria associated with lymph nodes. He tabulated 4 distinct groups, which when analyzed showed 8 different groups according to fermentation reactions. One of the groups included chromogenic types, chiefly orange-red. This species I have named C. aurantiacum. An anaerobic diphtheroid occurring in lymph nodes was described by Torrey and named B. lymphophilus. It is slender, pleomorphic, gram-positive, from 0.5 × 1.2-3.2 mikrons. It is not acid-fast, occurs singly or in pairs, is nonmotile; stab cultures in 0.5% agar show rods of irregular morphology and uneven staining. On Loeffler's medium, raised white growth occurs. In dextrose, high acid is produced, even to the amount of 10 +. It is markedly aciduric and acidophilic. Glycerol is also fermented, but lactose, mannite, inulin and dextrin are not attacked. No gas is produced. In my studies, several strains were isolated which exhibited anaerobic preference for a few generations only, but were not like Torrey's strain either with respect to anaerobic requirements or unusual acid-production in any one carbohydrate. Torrey's results are of special interest in showing that there are several types of diphtheroids commonly present in the lymph nodes of Hodgkin's disease, none of which has definite pathogenic properties or can be accepted as the cause of the disease. In this respect he confirms the findings of Bloomfield and myself.

EYE DIPHTHEROIDS

Diphtheroids isolated from the eye were found to vary in cultural behavior. Three strains were obligate aerobes and produced only a very slight amount of acid in dextrose with 0.5 c c N/20 acid maltose. Seven other strains, facultative, produced slight acid in the same sugars. One strain grew slightly, if at all, on agar and favored Loeffler's serum. All of these grew rather delicately on ascitic agar and serum. At room temperature growth was slow. The organisms were large, clubbed and striated. The type C. xerosis prevailed.

NASAL DIPHTHEROIDS

Ten strains were isolated from nasal discharges, mostly in persons with cold in the head. Of these, 5 strains were characterized by slight acid formation in dextrose with marked acid in maltose. The remaining 5 were of 2 types, one which produced slight acid, the other abundant acid in dextrose and maltose. On Loeffler's serum, growth was abundant, white and glistening. Stained with anilin dyes, the organisms appeared short and thick with larger striped forms predominating. They are gram-positive, facultative, nonmotile, do not form spores, are not acid fast and grow well in sugar broths. At room temperature moderate to abundant growth takes place. The type C. striatum prevailed.

DIPHTHEROIDS FROM ASCITIC FLUIDS

Thirty specimens from cases of cirrhosis and tuberculosis were studied anaerobically and aerobically for diphtheroid organisms. No strict anaerobes were found. For anaerobic cultures, Buchner tubes (Zinsser's modification*) were used. Three strains were isolated. All are gram-positive, nonmotile, do not form spores, are not acid-fast, are facultative. Two strains grow very rapidly at room temperature, a third does not. One liquefies gelatin and gives rise to an offensive odor after several days. In broths, the following results were noted (litmus used as indicator).

* A piece of absorbent cotton is pressed down over the pyrogallol to prevent rapid mixing with the NaOH.

Strain	Dextrose	Lactose	Saccharose	Maltose	Mannite	Dextrin	Raffinose
9	+	—	+	+	+	+	—
42	—	—	—	—	—	—	—
43	—	—	—	—	—	—	—

Strain 43 liquefies gelatin and produces a putrid odor. This strain, practically conforms to the description given by Graham Smith to a liquefying diphtheroid which he named B. diphtheroides liquefaciens. Motility is lacking, however. As was mentioned before, it does not seem as if this property is peculiar to the group with which we are dealing. The organism isolated from ascitic fluid is slender, curved and appears granular. It does not form spores, is facultative, grows abundantly with confluent mass on agar at room temperature and at 37 C. and gives off a decidedly putrid odor. On Loeffler's serum it produces a heavy white, moist and glistening layer. It is suggested that the name C. putidum sp. nov. be given to this species. Strain 9 is characterized by its wide range of fermenting power. It is a very small rod, not much larger than B. influenzae. On first isolation, club-shaped forms predominated. It is gram-positive, nonmotile, does not form spores, is facultative, forms acid in dextrose, saccharose, maltose, mannite and dextrin. It grows abundantly at room temperature and at 37 C. On Loeffler's serum, abundant growth occurs. It does not liquefy gelatin. C. ascites sp. nov. is proposed as a name for this diphtheroid. Strain 42 is a small gram-positive organism, nonmotile, forms no spores, grows moderately on ascitic agar at 37 C. In the early generations this organism showed a decided preference for anaerobic conditions. No growth takes place at room temperature after 10 days. It does not liquefy gelatin and does not ferment sugar broths. Grows best in raffinose broth. This seems to be the most selective medium for obtaining abundant growth. All of the diphtheroids studied grew moderately well and for the most part abundantly in the presence of this trisaccharid when other sugars used for fermentation studies showed little or no growth other than a sediment. C. delicatum sp. nov. is suggested as the name for this species (see blood diphtheroids).

In the early generations this organism showed a decided preference for anaerobic conditions.

A peculiarity noted in the isolations from ascitic fluids was the fact that the first generation always had larger clubbed and knobbed forms. In succeeding subcultures only very small diphtheroids were seen.

DIPHTHEROIDS IN BLOOD CULTURES

Two strains were isolated from blood cultures in cases of pneumonia. In the first generation these showed larger clubbed forms and small rods. Growth is delicate. The organisms are small gram-positive, nonmotile, facultative and produce very slight acid in dextrose or maltose but as a rule leave all sugars unfermented. Gelatin is not liquefied and growth at room temperature is negative after 10 days. On Loeffler's serum moderate growth occurs. These diphtheroids have been identified with C. delicatum.

SKIN DIPHTHEROIDS

Hine working with 34 strains of diphtheroids found a group which was associated with skin wounds or diseased cavities opening on to or near the surface. Strains of this group were found to acidify dextrose, saccharose usually and less often maltose. The author did not describe these organisms. That diphtheroids can be found on the skin very readily is well established. Judging by the amazing number of sources from which the organisms of this group may

be isolated, it seems highly probable that we have to deal with saprophytes which abound everywhere like numerous species of the Coccaceae group and which gain entrance into the body through the various openings, both normal as well as pathologic.

In the course of this study 4 strains were isolated from an anal pus pocket and from the skin on the neck. Two of these strains which will be described below are in accord with those mentioned by Hine; the other 2 differ sufficiently from these in fermentative and other cultural characters to warrant their acceptance as other species.

(1) Skin Diphtheroid: On Loeffler's serum, abundant dull white growth is produced. On agar delicate growth takes place. Gelatin is not liquefied. At room temperature little growth is observed after 1 week. The organism is gram-positive, nonspore forming, nonmotile, facultatively aerobic, small, ovoid and stains readily. Slight acid is produced in dextrose and maltose, the latter being more strongly acidified. It is avirulent. The name C. epidermidis sp. nov. is suggested for this species.

(2) Anal Pus Pocket: On Loeffler's serum abundant, moist and glistening growth occurs. On agar, heavy glistening growth takes place in broth, uniform clouding. Ferments dextrose and maltose vigorously and usually saccharose. It is gram-positive, facultative, nonmotile, does not liquefy gelatin, does not form spores, and grows abundantly at room temperature. It is avirulent. To this species the name C. suppuratum sp. nov. is given. Similar strains have been observed by Trumpp in empyema and by Hine in a suppurating neck gland. The organism in no way appears to be associated with suppuration except as a secondary invader.

DIPHTHEROIDS FROM BRAIN

One strain was isolated from the brain in a case of meningitis. The organism is gram-positive, ovoid, or coccoid in appearance, nonmotile, nonspore forming. It grows very rapidly at room temperature and at 37 C. on ordinary agar. On Loeffler's serum, growth is white and moist. It is avirulent for guinea-pigs and for rabbits injected intraperitoneally and subdurally. Vigorous acid production takes place in dextrose, lactose and maltose. Growth in broth is abundant. Gelatin is not liquefied. Ortmann has reported a diphtheroid isolated from the brain in a case of meningitis but has given no description of the organism. It is purely a secondary invader. The name C. cerebralis sp. nov. is suggested for the species.

DIPHTHEROIDS FROM THE APPENDIX

One strain was obtained from this source. There was slight suppuration of the appendix. The organism grows moderately on Loeffler's serum. On agar moderate growth occurs. Few clubbed and barred types were observed in the first generation and in the next few transplants. Neisser's granules are present. The organism is fairly large, nonmotile, gram-positive, does not produce spores and does not liquefy gelatin. At room temperature slight growth occurs. In dextrose, lactose, saccharose, maltose and dextrin slight acid is formed. Heavy flaking with sediment, but no clouding, occurs in the broths mentioned.

Hine has obtained a diphtheroid from an appendix but has not described the organism. It is a saprophyte and a secondary invader. The name C. flocculens sp. nov. is suggested for this species.

In all, diphtheroids were obtained from 21 different sources and these fell into 12 distinct fermentation groups, as shown in Table 3. The greatest number of organisms comprised the group of nonfermenters and here is to be noted the fact that of 13 strains from Hodgkin's disease, 8 came under this head. However, little significance need be attached to this point, inasmuch as strains from Hodgkin's glands fell into four other groups besides this one. In the same way, it is to be noted that strains from the eye and nose fall into 4 and 3 different groups, respectively.

TABLE 3

SOURCE AND FERMENTATIVE REACTIONS OF DIPHTHEROIDS

Source	Dextrose	Dextrose, Saccharose	Dextrose, Maltose	Dextrose, Lactose, Saccharose, Maltose	Dextrose, Saccharose, Maltose, Dextrin	Dextrose, Saccharose, Maltose, Mannite, Dextrin	Dextrose, Saccharose, Maltose	Dextrose, Maltose, Dextrin	Dextrose, Lactose, Maltose	Dextrose, Maltose, Lactose, Dextrin	Dextrose, Lactose, Saccharose, Maltose, Mannite, Dextrin	N m
cne (pustule)	1	..	..	..	..	..	..	2	..	..	..	
ppendix	..	..	..	..	..	..	..	..	..	..	..	
scitic fluid	..	..	..	..	..	1	..	..	..	..	..	
ile	..	..	..	..	..	..	..	..	..	..	..	
lood	..	..	..	..	..	..	..	..	..	..	..	
rain	..	..	..	..	..	..	..	..	1	..	..	
ar	..	..	..	..	..	..	..	..	..	..	2	
ye	2	2	1	..	..	..	..	..	..	..	..	
ymph nodes, Hodgkin's	..	1	1	..	1	..	2	..	..	..	..	
ymph nodes, leukemia	..	..	..	..	..	..	..	..	..	..	..	2 ke
ymph nodes, pseudoleukemia	..	..	..	1	..	..	..	..	..	..	..	
larrow	..	..	..	..	..	..	..	..	..	..	..	
ose	4	1	..	..	..	..	..	..	..	..	..	
us (anal pocket)	..	..	1	..	..	..	2	..	..	..	..	
onsil	..	..	..	..	..	..	..	1	..	..	..	
rine	..	..	..	..	..	..	..	..	..	1	..	
lorse, calf, sheep (Preisz Nocard B.)	..	..	3	..	..	..	..	..	..	..	..	
nknown	..	..	2	..	..	..	..	..	..	..	..	
Total	7	4	8	1	1	1	4	3	1	1	2	

It becomes evident that neither the source nor the cultural characters serve to distinguish the supposed cause of Hodgkin's disease from numerous organisms which may be regarded as harmless saprophytes; also that diphtheroid organisms comprise a greater number of groups than hitherto supposed.

There appears to be a definite correlation between fermentative properties and source with regard to those organisms which were isolated from the nose and throat. These are characterized by relative inability to ferment carbohydrates. Acid formation in dextrose occurred but 6 times of 19; in dextrose and saccharose, 3 of 19; in

dextrose and maltose, but 1 in 19, and of the entire number of strains more than half failed to ferment any sugars. Acid formation, therefore, is limited in its range, never exceeding 2 carbohydrates and showing weak splitting action in general.

CLASSIFICATION

Classification from the cultural standpoint is difficult because of the manifold variations which are observed. The resolution of all diphtheroids into a few distinct "types" such as suggested by Morse is possible only for a limited number of organisms from a limited number of sources. Lehmann and Neumann classify "pseudodiphtheria" from eye and nose as Coryneb. pseudodiphtheriticum and Coryneb. xerosis, including diphtheroids from both sources under each heading.

More recent work such as that of Plotz on typhus fever (C. typhi-exanthematici) indicates that the diphtheroid group may possess pathogenic species which are far removed from the type C. diphtheriae. The organism described by Plotz and his co-workers is an obligate anaerobe with characteristic serum reactions. Some diphtheroids might be classified from the viewpoint of human and animal diseases, and particularly striking cultural characters such as oxygen requirement and sugar fermentations. C. acnes is anaerobic and might very well go into this group of anaerobes. Here also belong those "oxygen tension" diphtheroids which are not anaerobic in the usual sense of the word, but which grow beneath the surface in solid mediums. The property of gelatin liquefaction, not hitherto recognized as a diphtheroid character, suggests another subgroup.

With these points in mind the following classification is offered. An effort has been made to group the species logically but it is evident that relationships between different types do not always permit the most desirable grouping. Certain characters were used as a basis for arranging the distinct species under a chosen type. These will become evident as the tabulation is studied. In the key given here, all of the known diphtheroids have been reviewed and certain new species added.

CLASSIFICATION OF DIPHTHEROIDS

Group 1.—Diphtheria. Type C. diphtheriae (Klebs-Loeffler).—Nonmotile, nonsporeforming, gram-positive, does not liquefy gelatin, shows polar bodies in young cultures. Aerobic and grows between 19 and 42 C. Acid produced in dextrose, maltose and dextrin. Produces

toxin and gives rise to clinical symptoms known as diphtheria, for which a specific antitoxin exists.

C. pseudodiphtheriae sp. nov. (Fig. 10). — Avirulent organism, morphologically and culturally like C. diphtheriae but produces no toxin and exerts no action when injected into animals. Isolated from hypertrophied tonsils.

GROUP 2.—Pseudodiphtheriticum. Type C. pseudodiphtheriticum (Hofmann-Wellenhoff.)—Nonmotile, nonsporeforming, gram-positive, does not liquefy gelatin. It is shorter and thicker than the C. diphtheriae, but variations such as knobbed, clubbed and barred forms occur. It is avirulent and produces no toxin. In carbohydrates, no acid is produced.

C. ceruminis (Graham Smith) Eberson.—Differs somewhat from C. pseudodiphtheriticum in morphology, as well as in its behavior in broth which remains clear, with sediment. In carbohydrates, no acid is produced. It is avirulent. Isolated from normal ear.

C. delicatum sp. nov. (Fig. 6).—Avirulent strains from ascitic fluids and blood culture. The organism is small and delicate with a decided preference for anaerobic conditions in the early generations. Some strains form traces of acid in dextrose or maltose, but usually ferment none of the sugars.

GROUP 3.—Xerosis. Type C. xerosis (Neisser and Kuschbert).—Nonmotile, nonsporeforming, gram-positive, does not liquefy gelatin. Resembles C. diphtheriae closely morphologically. Acid is produced in dextrose, saccharose and maltose. The organism can be isolated from normal as well as diseased eyes. Growth solid medium is rather delicate.

C. epidermidis sp. nov. (Fig. 7). — Gram-positive, facultatively aerobic, non-sporeforming, does not liquefy gelatin. It is small ovoid in form. Little growth takes place after one week at temperature below 25 C. On Loeffler's serum abundant, dull white, growth. Slight acid is produced in dextrose and maltose. It is avirulent. Isolated from skin and pus pockets.

C. suppuratum sp. nov. (Figs. 3, 4).—Gram-positive, nonsporeforming, does not liquefy gelatin. Heavy glistening growth on agar and on Loeffler's serum. Ferments dextrose and maltose vigorously and usually saccharose. Isolated from pus pockets and abscesses. Avirulent.

C. auris (Graham Smith) Eberson.—Gram-positive, nonsporeforming, does not liquefy gelatin. Long barred or granular, often clubbed. Grows scantily on agar. Acid in dextrose and maltose, but not saccharose. It is often virulent for guinea-pigs. Isolated from ear.

C. cerebralis sp. nov. (Fig. 11).—Gram-positive, ovoid or coccoid, nonmotile, nonsporeforming, does not liquefy gelatin. Grows rapidly at room temperature and at 37 C. on agar. On Loeffler's serum, growth is white and moist. Vigorous acid production in dextrose, lactose and maltose. Avirulent for guinea-pigs and rabbits. Isolated from brain.

GROUP 4.—Nodosum. Type C. nodosum (Migula) Eberson.—Large, clubbed forms markedly segmented. Does not form spores, is gram-positive, nonmotile, does not liquefy gelatin, white growth on agar. Acid in dextrose, saccharose, maltose, and dextrin. Found frequently in urine and the urogenital tract. Avirulent.

C. acidum sp. nov. (Figs. 1, 2).—Gram-positive, nonmotile, small rods, with abundant white, glistening growth on agar. No spores, is aerobic and does not liquefy gelatin. Produces large amounts of acid in dextrose, lactose, saccharose, maltose, mannite and dextrin, but does not attack raffinose. Isolated from urine. Avirulent.

C. ascites sp. nov. (Fig. 8).—Gram-positive, small rod with numerous club-shaped forms on first isolation. Nonmotile, nonsporeforming, does not liquefy gelatin, grows abundantly on agar and Loeffler's serum. Forms acid in dextrose, saccharose, maltose, mannite and dextrin. Avirulent. Isolated from ascitic fluid.

C. flocculens sp. nov. (Fig. 13).—Gram-positive, nonmotile, clubbed or barred forms, does not form spores or liquefy gelatin. The organism grows moderately on agar and Loeffler's serum at 37 C. At room temperature slight growth. Neisser's granules present. Produces acid in dextrose, lactose, saccharose, maltose and dextrin. Heavy flaking with sediment without clouding in broths. Isolated from a suppurating appendix.

GROUP 5.—Pigment-forming Diphtheroids. Type C. hoagii (Morse) Eberson.—Medium sized bacterium with solid, barred and wedge-shaped forms with abundant but imperfect granules. On serum a heavy, confluent, glistening growth with a characteristic, salmon-pink color. Gram-positive, forms no spores, does not liquefy gelatin, nonmotile. Produces acid in dextrose and saccharose, but not in maltose.

C. aurantiacum sp. nov. (Fig. 5).—Gram-positive, small and coccoid, nonmotile, nonsporeforming, does not liquefy gelatin, not acid-

fast. Grows abundantly on agar at room temperature and at 37 C. and produces a bright orange pigment. No polar bodies or granules. Grows well in sugar broths and produces acid in dextrose and maltose. Pigment production in 24 hours under aerobic and anaerobic conditions at 18-25 C., but at 37 C. pigment in this length of time only under aerobic conditions. Under anaerobic conditions, at 37 C. a trace of pigment was evident after 10-14 days.

C. glandulae sp. nov. (Fig. 9).—Small ovoid or coccoid bacterium. Grows abundantly at room temperature or at 37 C. with a heavy creamy consistency and brownish tinge. Gram-positive, nonmotile, nonspore forming, does not liquefy gelatin. Grows abundantly in sugar broths with uniform clouding. On Loeffler's serum abundant white glistening growth occurs, changing to a brownish color after a few days. Produces acid in dextrose, saccharose and maltose vigorously and slight acid in dextrin. Raffinose neutral. One strain on first isolation from the gland on blood plates showed a remarkable likeness to B. tuberculosis in character of growth; the colonies were dry and heaped up and friable. This peculiarity did not persist in later transplants. Antiformin treatment showed these diphtheroids to be nonresistant. They were not acid-fast. Isolated from glands of Hodgkin's disease.

C. striatum (Chester) Eberson.—Thick form, with clear cut bars. Large and irregular granules visible. Heavy growth on serum with pigment from white to yellow. Ferments dextrose and maltose, but not saccharose. Gram-positive, does not form spores, nonmotile, does not liquefy gelatin. Found in normal nasal mucus.

C. segmentosum nom. nov.—Nonmotile, gram-positive, nonspore-forming, does not liquefy gelatin. Polar bodies demonstrable. In broth, growth is clear with sediment. Slight acid is produced in dextrose. Avirulent, from nasal mucus.

GROUP 6.—Gelatin-liquefiers. Type C. putidum sp. nov. (Fig. 12). —A slender, curved granular bacterium. It produces no spores, is facultative, grows abundantly with confluent stringy mass on agar at room temperature and at 37 C., giving off a decidedly offensive odor. On Loeffler's serum, a heavy, white, moist and glistening mass. It forms no acid in dextrose, lactose, saccharose, maltose, mannite, dextrin and raffinose. Isolated from ascitic fluid.

GROUP 7.—Anaerobic Diphtheroids Associated with Specific Clinical Manifestations. Type C. typhi-exanthematici (Plotz).—Small, pleomorphic, gram-positive organism, not motile, not acid-fast. Most

of the bacteria are straight, some slightly curved, coccoid forms also occur. The ends are slightly rounded or pointed. Nonspore forming. It is an obligate anaerobe and can be isolated on special glucose mediums containing ascitic fluid of a definite specific gravity. Produces acid in dextrose, galactose, maltose and inulin. Found in typhus fever with regularity; specific serum reactions; pathogenic.

C. acnes (Gilchrist) Eberson.—Gram-positive, small organism. resembling C. typhi-exanthematici. Stains unevenly; anaerobic, but many strains grow aerobically after continued cultivation. Surface smears fail to show growth with material from pustules, whereas deep stab cultures and heavily inoculated unbroken pustules always give growth. Mediums with large amounts of acid especially adapted for isolating this organism which produces acid in dextrose, saccharose (slight), maltose, mannite and inulin. Raffinose is fermented by some strains. Pathogenic for mice and gives rise to characteristic lesions.

GROUP 8.—Pyogenic Diphtheroids in Animals. Type C. pseudotuberculosis (Preisz) Eberson.—Gram-positive, nonmotile, club-shaped. On serum heavy moist growth with golden-yellow pigment. Toxin production is attributed to the organism and it is fatal for guinea-pigs, rabbits, goats and sheep. Blood agar with a minimum amount of fermentable carbohydrate shows hemolysis. Acid in dextrose and maltose, but not glycerin. Produces suppurative processes in the lymphatics.

C. pyogenes (Glage).—Gram-positive, nonmotile, club-shaped or cylindrical. Does not grow at room temperature. Is facultatively aerobic, produces no spores and is hemoglobinophilic. Pathogenicity doubtful. No acid produced in dextrose or glycerol.

C. vaccinae (Galli-Valerio). — Gram-positive, nonmotile, club-shaped, coccoid, segmented or branched. Facultatively anaerobic, nonspore forming. On serum, growth abundant; pale yellow or orange-yellow pigment produced; gelatin not liquefied. Pathogenic for guinea-pigs, mice and rabbits. Associated with vaccine pustules in calves.

GROUP 9.—Anaerobic Diphtheroids Associated with Diseased Lymph Glands. Type C. lymphophilus (Torrey).—Slender, pleomorphic, gram-positive, nonmotile; markedly aciduric and acidophilic; ferments dextrose and glycerol; avirulent. From lymph nodes in Hodgkin's disease.

DISCUSSION

From this study, which is far from complete, it is evident that the sources of diphtheroid organisms have not yet been exhausted. That many strains are yet to be described is a foregone conclusion. The

bacteriologc study of body fluids and glands and of secondary invaders in common diseases will undoubtedly give us material for ultimate classification. Apart from Morse's biometric study of the diphtheria group, no other classification has been presented. In the light of the material given here it seems as if Morse's classification on the basis of chromogenesis, vigor of growth and fermentative properties is not entirely adapted to the great number of strains which have already been described and which are forthcoming. Serologic studies as a means of classification in this group of bacteria have not been worked out sufficiently to merit much discussion; the little work done has not been of a very searching nature. It seems that here are the most promising possibilities. Agglutination has been tried by Nicholas, Nicolle, Landsteiner, Bruno, Lubowski, Lesieur, and Fraenkel with varying results, which were not specific. The consensus of opinion seems to be that the organisms do not produce agglutinating substances in the serum. Complement fixation has not been sufficiently studied. The results of Morse show that cross-reactions may be obtained for the diphtheroid antigens and that C. diphtheriae does not fix with any of the diphtheroid antigens. The results, however, are not striking enough to be conclusive. The method of preparing antigen undoubtedly will modify the results in such tests. Ground bacteria were used and specific serum obtained by the injection of formalized suspensions of whole organisms. Perhaps Olitzky's method for complement fixation in typhus fever would be preferable. According to his technic, nonspecific fixation is prevented by the use of Berkefeld filtrates of autolyzed bacteria. It is hoped that by means of group reactions the diphtheroids may be classified definitely. Precipitation tests suggest good possibilities. The reaction is exceedingly delicate and the organisms lend themselves to ready disintegration and extraction.

II. SEROLOGICAL

EXPERIMENTS WITH HODGKIN'S STRAINS

In order to determine the identity of cultures isolated from cases of Hodgkin's disease, two methods were utilized—agglutination and complement fixation. The first method enables us to identify the specificity of the organisms by means of serums obtained from patients suffering with the disease as well as by serums prepared with the different strains of bacteria isolated. Obviously, if the C. Hodgkinii is the cause

of Hodgkin's disease we ought to be able to show a definite relationship between serums from patients and organisms isolated from these and other cases. By the delicate method of complement fixation we may detect similar antigenic substances and establish the identity, if such there be, of the several strains isolated.

AGGLUTINATION

Four serums, obtained from 4 authentic cases of Hodgin's disease were studied. Eleven strains of bacteria were tested, of which 8 were isolated from glands in Hodgin's disease and the remaining 3 from a case of leukemia, ascitic fluid in cirrhosis and blood.

Forty-eight-hour agar cultures were suspended in salt solution and emulsions made up to approximately the same density. These were shaken with glass beads for thirty minutes and then centrifugated for 5 minutes at low speed to throw down coarser particles. Equal volumes of the different serums (diluted with NaCl solution) were mixed with the bacterial suspensions in a hanging drop. The preparations were well sealed with vaselin and incubated at room temperature for 1 hour, then at 38 C. for another hour, when the results were read.

The results were entirely negative and confirm the results of Fox.

COMPLEMENT FIXATION

In these experiments the aim was to show by tests for cross fixation what relationship, if any, existed between the different types of bacteria isolated from cases of Hodgkin's disease. The cultures, as in the preceding, were obtained from Drs. Bunting, Yates and Rosenow and from cases studied by me. These strains have been described fully in Part I.

Four strains were used in the production of immune serums from rabbits. These cultures are designated 2, 8, 13 and 57. No. 2 was isolated by Dr. C. H. Bunting from a patient who had a very acute form of Hodgkin's disease; it fermented dextrose and saccharose with acid. No. 8 was sent to me by Dr. E. C. Rosenow, and labeled B. hodgkinii; it produced acid in dextrose, maltose, saccharose, and dextrin. No. 13 was obtained from the same source; it fermented dextrose and maltose. No. 57 was isolated by me from a case of acute Hodgkin's disease. This organism fermented none of the carbohydrates and was identical in this respect with a strain (No. 1) obtained from Dr. Bunting.

Healthy rabbits, weighing about 1,600 gm., were systematically treated with each of these cultures heated to 58 C. for 30 minutes. Injections were made with increasing doses every week for a period of 6 weeks and the serums drawn 10 days after the last injection.

Antigens were prepared by grinding to a fine powder the flocculent precipitate obtained from a mixture of bacteria with an excess of absolute alcohol. The powder was dried in vacuo and then ground in a mortar with solid NaCl. Subsequently sterile distilled water was added to isotonicity and the anticomplementary unit determined.

Complement was obtained from normal guinea-pigs and titrated in the usual way. Sensitizer was prepared with thoroughly washed sheep blood cells injected into rabbits and the unit determined, after which a 5% suspension of sheep cells was sensitized with 2 units of the amboceptor. Readings were taken after the tubes were incubated for 15 minutes in a 37 C. water bath, and 8 hours in the ice-chest.

The results showed no correspondence among the separate strains and hence prove them to be nonidentical, a fact which confirms the cultural differences observed in the same organisms. Fox failed to obtain fixation with the serum of a patient and a strain isolated from the glands.

TABLE 1

RELATIONSHIP OF TYPE DIPHTHEROIDS

Serum Anti-hoagii

Dilution	Before Absorption			Absorbed with Pseudodiphtheriae		Absorbed with Flavidus (Mellon)	
	Hoagii	Pseudo-diph-theriae	Flavidus (Mellon Strain)	Hoagii	Pseudo-diph-theriae	Hoagii	Flavidus (Mellon)
1-20	++	+	++	++	—	++	—
1-40	++	+	++	++	—	++	—
1-80	++	+	+	++	—	++	—
1-160	++	±	+	++	—	++	—
1-320	++		+	++	—	++	—
1-500	++		±	++		++	
1-1,000	+		—	+		±	
1-1,500	+			±		—	
1-2,000	±			—		—	

Serum Anti-pseudodiphtheriae

Dilution	Before Absorption			Absorbed with Flavidus (Mellon)	
	Pseudo-diphtheriae	Flavidus (Mellon)	Hoagii	Pseudo-diphtheriae	Flavidus (Mellon)
1-20	++	+	+	++	—
1-40	++	+	+	++	—
1-80	++	+	±	++	—
1-160	++	±	—	++	—
1-320	++	—		+	
1-500	++	—		±	
1-1,000	+			—	
1-1,200	—				

Serum Antiflavidus (Mellon)

Dilution	Before Absorption		Absorbed with Flavidus (Morse)	
	Flavidus (Mellon)	Pseudo-diphtheriae	Flavidus (Mellon)	Flavidus (Morse)
1-20	+	±	+	—
1-40	+	—	+	—
1-80	+	—	+	—
1-160	+	—	+	—
1-320	+	—	+	—
1-500	+	—	+	—

AGGLUTININ ABSORPTION TO DIFFERENTIATE TYPE DIPHTHEROIDS

The literature on diphtheroids, to my knowledge, does not contain any reference to agglutinin absorption as a method for showing relationships which might exist among overlapping, closely-related types. In view of the fact that the differences which characterize the accepted

forms are so slight, bacteriologically speaking, such a method suggested possibilities for more definite classification.

The strains studied were obtained from the Museum of Natural History and were marked as follows: C. hoagii, C. flavidus (Mellon), C. pseudodiphtheriae, C. xerosis, C. enzymicus (Mellon), and C. flavidus (Morse).

Agglutinating serums were prepared in rabbits by giving intravenous injections of fresh agar cultures heated at 56 C. for 30 minutes. Increasing doses were given every week until a whole culture was reached. The animals gained weight throughout treatment and were bled 10 days after the last injection. Preliminary titrations were made with each of the serums against homologous and heterologous strains, and double absorption of each serum was made with organisms which evidenced some affinity for the agglutinins present.

The results of the absorption experiments indicate that C. hoagii is more inclusive in its antigenic elements, since it possesses group agglutinins for all the other strains, excepting C. xerosis. C. flavidus (Mellon) has no group agglutinins for any of the others and appears to be highly specific. The results vary somewhat as compared with Morse's complement fixation results. Mellon's flavidus shows no group agglutinins at all, although Morse obtained partial cross fixation with her strain. It appeared likely that the former culture sent to the Museum of Natural History as a "B. flavidus," isolated by Mellon, was not a flavidus type. When its homologous serum was absorbed with C. "flavidus" (Morse), the specific agglutinins remained unchanged. That complete absorption by the heterologous strain took place is evident from the table. Macroscopically, the Morse culture differs markedly on plain agar from the Mellon strain. The latter develops a dull, dry growth, with a tendency to flocculate readily in NaCl solution, whereas the former is moist and glistening in its growth. In carbohydrates the comparative results were as shown in Table 2.

TABLE 2

COMPARATIVE RESULTS

	Flavidus (Morse)	Flavidus (Mellon)
Dextrose	3.3 c c	2.0 c c
Maltose	2.0	1.7
Saccharose	2.5	1.4
Glycerol	3.3	2.6

The figures represent the amount of N/20 NaOH required to neutralize 5 c c of broth culture incubated 1 day at 37 C. Growth was heavy in both sets. The Mellon strain gave a flocculate growth and the Morse culture was uniformly clouded.

The relative rate of acid-production by both strains is obviously different. Prolonged cultivation may give somewhat different results, but it has been shown by several workers that the maximum acid-

production in the usual carbohydrate concentrations is at a maximum after 24 hours. In any case the antigenic properties of both strains are not in agreement, and the cultural tests seem to bear out this difference. Mellon's flavidus may be a subspecies, but it differs from Morse's type too markedly to go by the same species name.

Closer agreement exists in the case of C. hoagii, for which we find on the one hand neither group agglutinins nor cross-fixation for C. hofmanni and C. xerosis, while, on the other hand, there are both cross-fixation and group-agglutination with the flavidus antigen. C. hofmanni shows group agglutinins for C. hoagii and C. flavidus (Mellon), but not for C. xerosis.

TABLE 3

AGGLUTINATION TESTS AND COMPLEMENT FIXATION STUDY

Antiserum	Antigen			
	C. flavidus	C. hoagii	C. xerosis	C. hofmanni
C. flavidus	0.025 (500)	0.1 (—)	0.1 (—)	0.4 (20)
C. hoagii	0.05 (500)	0.025 (2000)	... (—)	... (160)
C. hofmanni	(160)	(80)		(1000)

The figures and signs enclosed in parentheses represent the highest dilution in which partial agglutination occurred, and the decimals indicate the amount of antigen in mg. which was necessary for fixation with the different serums. Absorption experiments with a strain of Morse flavidus omitted.

There is no apparent correlation between the reactions in carbohydrates and the antigenic properties. Nonspecific agglutinins appear to be readily absorbed, whereas the specific agglutinins are left intact.

Morse's complement fixation study and the group agglutination tests are shown in Table 3.

III. PLEOMORPHISM OF DIPHTHEROIDS, MUTABILITY OF TYPES, AND A METHOD FOR DETERMINING MUTANTS

VARIABILITY OF BACTERIA

Bacteria are unicellular organisms which are produced from other like organisms by the process of fission—an act in which the entire parent substance may be said to divide itself equally into two fractions, both of which constitute the resulting pair of bacteria. Under such conditions the offspring cannot possess what was not present in the parent. This, in essence, is the pure line concept of bacteria and implies the descendants from any single bacterial cell. The hereditary characteristics of such a biotype do not change as a result of changed

environmental conditions. In order to explain variations in bacterial types it is necessary to consider both Darwin's theory and that of deVries. The former conception is based on natural selection in which such variations as are better adapted to the struggle for existence will be perpetuated. The theory of deVries holds that the tendency toward variation in the germ plasm may give rise to permanent variations as opposed to fluctuating variations.

When bacteriology was in its infancy it was a popular notion that types could be transmuted readily from one into the other and back again. Strange as it may seem the idea has been reborn and given great impetus within recent years. Pleomorphism is highly developed among the diphtheroids and it is in this group that much confusion has arisen not only as a natural result of the well known overlapping of closely related types, but chiefly because of the ease with which apparently pure cultures of the organisms undergo changes in their morphology. In none of the work presented has the technic been of such a nature as to preclude error. Billings and Rosenow state that single colonies of diphtheroids from Hodgkin's disease "in dextrose agar which showed bacilli only in smears, yielded in subcultures a pure culture of staphylococci aerobically and forms of the bacillus either pure or in mixture anaerobically on the same medium. These facts suggest strongly that the associated staphylococcus is derived from the bacillus." Certainly, no evidence is given by the authors that the single colonies were pure. There is only one method whereby the fallacy may be precluded and that method was not used in this particular instance. It may or may not be true that the staphylococcus was associated with the bacillus, but the word "derived" is misleading. We know from common practice that a single colony even when obtained from highly diluted plate cultures, does not necessarily represent the descendants of a single bacterial cell. Such a mistake was made by Goodman when he claimed to have separated the diphtheria organism into an acid-producing and an alkali-producing type by the plate method, on the assumption that he had a pure line.

I shall deal exclusively with C. enzymicus described and named by Mellon. This organism exhibits the pleomorphism of most diphtheroids but is of especial interest because of the remarkable morphological changes the author claims to have produced. He was able to show a seeming relationship between the diphtheroids and the streptococci and described a method whereby he transformed the bacillary type of diphtheroid into a coccus and back again. The old question of sta-

bility of bacterial type comes up and it is essential that the cardinal points be investigated. Two methods of attack were followed in my studies with C. enzymicus: cultural and serological.

CULTURAL STUDY OF MUTATION

Two strains of C. enzymicus were studied. One was obtained from the Museum of Natural History and had been cultivated on plain agar for several months. Smears stained with Loeffler's methylene blue showed typical diphtheroid bacillary forms with the usual coccoidal or short rod and extremely small forms (Fig. 14). The second culture was sent by Dr. Mellon. This strain had been cultivated on blood agar for about 1 year and in smear preparations appeared to be practically free from coccoidal forms, showing large unevenly staining bacillary types, varying in size and morphology.

Museum Strain of C. Enzymicus.—A subculture was made on plain agar slants and the 24-hour growth examined. Stains were made from different areas and examined with a $^1\!/_{12}$ immersion, using a magnification of approximately 1,400. In all instances diplococci and coccilike organisms were seen grouped like staphylococci and at times singly. Careful examination showed clusters of bacillary forms in small numbers (Fig. 15).

The next step was to obtain a pure culture of the coccus type. Agar plates (meat infusion agar 1.0 to phenolphthalein) were streaked with a platinum loop touched to original culture and several discrete colonies fished. In all cases a single colony was used to prepare a second plate culture and from this generation of single colonies, other plates streaked successively until the 10th generation. Each generation was studied for morphology before seeding. Pure coccus forms were obtained in every case (Fig. 16). No difficulty whatsoever was experienced in procuring a coccus form from the original culture. The real hardship lay in trying to obtain the bacillary type in relatively pure culture, free from cocci.

It is a well known fact that blood and serum mediums exert a favorable influence on the diphtheroid bacteria, causing the development of large clubbed and barred forms. Taking advantage of this fact, and the fortunate "transformation" effected on plain agar, it becomes possible to demonstrate the mechanism of variability in diphtheroids.

Again using the original culture of C. enzymicus I transplanted it to sheep blood agar and examined the culture after 24 hours' incubation. Smears showed cocci and numerous bacillary forms. When subcultured on the same medium the bacillary forms had almost completely outgrown the cocci and a typical diphtheroid picture was seen. As generation after generation on this medium was studied, it was found that cocci disappeared, although not entirely. At times it required careful examination of the microscopic field to discern them among the larger barred and granular forms. A most interesting point was next observed. When subculturing on sheep blood, transfers were made to plain agar slants from the first generation on sheep blood medium. After 24 hours' incubation the agar tubes were found to contain cocci only. As progressive generations on blood medium were transferred to plain agar it became more and more difficult to suppress the bacillary types and to demonstrate the coccus. By the use of various mediums and numerous cross subinoculations it appeared quite clear that the selection of biotypes was dependent on the environment. These details are shown in Chart 1. Certain cardinal points may be observed by studying the diagram in which evidence is adduced

CHART 1

Effect of Various Mediums on Selection of Biotypes*

I Sheep blood agar C+++ B++ → I Loeffler C+++ B++ → I Sheep blood agar B+++ C+

↓ I Plain agar C++++

II Sheep blood agar B++ C+

↓ I Plain agar C++++

↓ II Plain agar C++++

↓ III Plain agar C++++

↓ IV Plain agar C++++

III Sheep blood agar B+++ C+

↓ I Plain agar C+++

↓ II Plain agar C++++

IV Sheep blood agar B+++ C+

↓ I Plain agar C++ B++ → II Plain agar B+++ C++++ (P. 5)

↓ III Plain agar B+++ C++++

↓ I Loeffler C++ B++

V Sheep blood agar B+++ C± → I Glucose agar C++++

↓ I Plain agar B+++ C++++ (see above)

VI Sheep blood agar B+++ C++++ (see above)

I Loeffler C+++ B++

↓ II Loeffler B+++ C+

↓ III Loeffler B+++ C++ → I Glucose agar C+++ B±

↓ IV Loeffler B++ C++

↓ V Loeffler B+++ C+

I Glucose agar C+++ B±

↓ I Loeffler C+++ B±

↓ II Loeffler B+++ C+

I Sheep blood agar B+++ C+

↓ II Sheep blood agar B++ C++

↓ III Sheep blood agar B+++ C+

II Sheep blood agar B++ C++ → I Glucose agar C+++ B±

↓ I Sheep blood glucose agar C++++

II Sheep blood agar B++ C++ → I Sheep blood glucose agar C+++ B±

↓ I Sheep blood agar C+++ B±

↓ II Sheep blood agar B++++ C++++ (P. 4)

* The Roman numerals indicate generations and the arrows subcultures. C=cocci; B=bacillary; ±=rare; +=few; ++=numerous; +++=practically pure culture; ++++=pure type.

that we are dealing with distinct biotypes side by side in a culture of bacteria. When conditions are optimum for a particular biotype, variations do not occur readily. A new type which may have been brought out in company with the predominating type by a change to a less favorable environment can be suppressed as conditions are restored to normal. It is to be noted, however, that prolonged cultivation under what were at the outset unfavorable conditions, often, if not as a rule, leads to adaptation, and it becomes impossible to obtain a single biotype.

This hypothesis is supported strongly by the following observation: A subculture on plain agar made from the 4th generation on sheep blood medium (bacillary forms predominating) developed a peculiar surface growth of very heavy opaque masses lying in a more or less translucent delicate growth which composed the greater part of the transplant. Microscopic examination of the heavy growth showed purely bacillary forms with isolated cocci, whereas the translucent area gave cocci only. Touching a platinum needle lightly to the heavy mass a second tube of plain agar was inoculated and exactly the same picture was obtained (Figs. 17, 18, and 19, etc.). This was observed on blood medium also (Chart 1). As to the microscopic appearance of the culture it was found that no hard and fast rule can be laid down regarding the character of the bacillary or coccus growth. On any medium the former developed moist and opaque while the latter grew more delicately and translucent or transparent. These characteristics were observed with the agar strain of C. enzymicus and are diametrically opposed to Mellon's description. The strain cultured on blood medium conformed somewhat to his statement, yet transfers to plain agar gave heavy, moist growth as well as the more delicate and translucent. On fresh Loeffler's medium or blood agar the coccus type could distinguished macroscopically from the bacillary form.

To settle the question whether the coccus form is a mutant it is necessary to isolate in pure culture the two biotypes which cultural experiments seem to reveal. A true mutation must appear in a pure line which has been observed over a definite period and shown to consist of a single biotype. As soon as reversion occurs when environmental conditions are restored to normal, we are no longer dealing with a mutation. Different types, to be genuine mutants, must have developed when conditions remained constant. According to Mellon the diplococcus form of C. enzymicus when obtained from the barred, long bacillary type, remains quite constant, and although it is possible, as he states, to cause it to assume a bacillary form again, this is accomplished with great difficulty. It is obvious that these characteristics are not those of a true mutant, since the coccus, according to the author, is not obtained until conditions are changed and since it may be transformed to the bacillary type although this is attended with difficulty. Mellon's view is that the organism is extremely pleomorphic and he disregards the likelihood of the coccus being a mutant or a distinct biotype in his original pure culture. It is difficult, admittedly, to prove that the coccus is an entity in the original culture, yet it can be done, especially since there are so many factors in favor of this conception. Mellon goes on to describe the source of his culture and says in part "the rabbit had received intravenous injections of the strain in bacillary form and was recovered from the gallbladder in long chains of streptococci." Is pleomorphism the only possible explanation for this strange transformation? It is just as plausible and simpler to account for this by selective action on the part of the organs for the coccus biotype.

Experiments with Mellon Strain Cultivated on Blood Medium.—This culture, obtained directly from Dr. Mellon, was morphologically like a typical diphtheroid containing barred, granular, split and clubbed forms, and was relatively free from very minute ovoid bacteria, so far as could be revealed by a routine smear examination. The strain had been cultivated on blood agar for about 1 year and showed well-developed adaptability to this medium.

Study with Pure Lines of C. Enzymicus Bacillary Type

Sheep blood dextrose agar plates were smeared with a platinum loop lightly inoculated with the original culture. After 24 hours' incubation at 37 C. single colonies were taken for seeding a second series of plates. This process was repeated for 10 generations, using in each instance but 1 colony for the subculture. A single colony, when examined in a smear, always showed the typical bacillary appearance, yet was not "pure" insofar as type was concerned. From 40 to 50 separate colonies selected from each generation were studied microscopically and invariably ovoid and very small coccoid forms could be found in goodly numbers. By carefully examining numerous fields the interesting fact is revealed that a small diplococcus or coccus type is present. Painstaking study of very many microscopic fields is necessary before passing judgment on the presence or absence of these types, but they can be found none the less. Whether or not these diplococci are appearances caused by unusually short ovoid or coccoid forms, I cannot say, since the line of demarkation between these types is not marked, but, as seen, they were distinctly spherical and appeared in pairs. Although the contrast was sufficiently striking with a magnification of about 1,500 diameters, a high power lens with magnification of 2,500 dispelled any doubt. Where two such organisms were united, one could distinguish the point of union from that which is observed in coccoid and small rod-forms. With these observations to go by, Mellon's contention regarding his bacillary culture becomes untenable, and it is perhaps inaccurate to say that "a culture of the C. enzymicus was prepared from a single colony whose antecedents had shown no diplococci." Theoretically as well as practically the culture is absolutely pure but we are not justified, under any circumstances, in assuming that a single biotype can be so obtained. It is not that it is difficult to obtain the bacillary form from the coccus, but that it is difficult, if not impossible, to get the bacillus without the coccus. Agar slants, inoculated with single colonies from various generations plated out according to the technic described, always contained cocci.

"Transition" of Bacillus to Coccus.—The technic of Mellon was repeated carefully in all details, Meat infusion veal glucose broth 1.6 to phenolphthalein was enriched with sterile rabbit serum to the amount of about 5 c.c. per liter. Flasks and tubes were prepared containing 30 c.c. and 10 c.c. of the medium, respectively, and tested for sterility before use. A single colony from the 8th generation of plates made with the bacillary types, previously described, was planted in a tube of this medium, incubated at 37 C. for 24 hours and then at 28 C. for 3 days. From this tube the sediment was transferred to a flask of the same broth and incubated at 37 C. After different intervals smears were made from supernatant fluid and sediment and examined. At no time was any change to a coccus observed, and the picture did not differ in any degree from that seen under ordinary conditions on agar slants. Before we can affirm that the morphologic appearance represents the actual mechanism of transformation, it is essential that a culture of cocci be obtained and propagated for several generations from material showing this "transi-

tional" picture. Repeating Mellon's technic I have not succeeded in demonstrating this and I find that his paper contains no evidence that such a postulate has been fulfilled. Subcultures were made on agar, after 12, 24, 30, 48, 72, and 96 hours, from individual flasks which had been seeded with the sediment obtained from tubes incubated at 25-28 C. for 3 days. In none of these transplants, incubated at 37 C. for 24 hours, was a culture of cocci obtained. There was, however, an abundance of extremely short forms—coccoids and some diplococcoids, but nothing to indicate that the bacillary type had undergone any definite change. When cocci are present, the picture is unmistakable and there can be no confusion of this type with the very short forms which look like diplococci (Fig. 26).

It is questionable whether the refined technic of Mellon is needed to demonstrate what is erroneously called a transformation. That all diphtheroid cultures show two distinct forms of organisms is beyond question and that these types may be seen under usual laboratory conditions, is also a matter of common experience. Great caution is necessary in interpreting a morphologic appearance as the actual process responsible for the occurrence of different forms in bacterial culture. Repeated subcultures gave negative results and at no time was it possible to show that complete disintegration had taken place. The "mucus-like débris" and "concentration of chromatin" described by Mellon was seen after 24 hours, not only in broth cultures made according to the special technic described, but also in agar slants grown under conditions to be given. Transfers from broth containing "disintegrated bacillary forms" naturally failed to give mucus-like shreds which are found in smears, but the explanation for this is very simple. Detritus can hardly be expected to grow in transplants. As for the "chromatin masses approaching each other in transplants until the figure cannot be told from a diplococcus," the fact remains that these forms only resemble diplococci. In order to study the effect of lower temperatures on the possible suppression of bacillary forms which might occur during the interval when the broth cultures are incubated at 25-30 C., a few experiments were performed with agar cultures.

Kinyoun's method for the staining of diphtheria was used. This stain gives most marked granular appearance and is best adapted to differentiating cocci from bacillary bodies with chromatin substance so disposed as to mislead one into taking these for true cocci. Agar slants were prepared from a bacillary culture of C. enzymicus and incubated at 37 C. for 24 hours. As usual, some coccus forms could be discerned in smears. The picture, however, was bacillary. Such cultures when incubated further at room temperatures, 21-23 C. for 72 hours, showed in smears numerous diplococci and a marked overgrowth of very short forms. Subcultures maintained at these temperatures always yielded the same results. When transfers were made to agar and incubated at 37 C., however, the diplococci became rare and finally could not be found at all. At 28 C. the effect on morphology was evident also. After 48 hours at this temperature, diplococci became numerous, and after 5 days they were present in practically pure culture. Transfers to agar incubated at 28 and 37 C. gave, respectively, numerous diplococci and short forms and few diplococci. Broth cultures, kept at 28 C. for 1 week, were found to contain numerous diplococci and extremely short forms which could not be distinguished readily from these.

Coccus Type.—A pure culture of cocci was obtained from the original diphtheroid strain, as described previously, and attempts were made to alter its morphology. The results of the experiments showed conclusively that when working with such a pure strain of cocci it is impossible to transform

it into a bacillus. In the serologic experiments described subsequently it is shown that the coccus is antigenically distinct from the bacillary type and that the extremely short types which are identical in appearance with cocci are merely altered bacillary forms. These "cocci," to be designated here as Strain 28, are antigenically the same as the long, granular rods.

Diphtheroid cocci were grown on Loeffler's serum for 27 generations, and no change was noted in morphology. The organisms remained cocci. On glucose serum medium the forms developed considerably larger and thicker, but were typical diplococci in outline. On agar of various H-ion concentrations ranging from 6.1-5.2, no change in form was observed. The use of potassium bichromate in broth containing various amounts of acid failed to alter the coccus.

A bacillary strain of C. enzymicus was cultivated on plain agar at 28 C. for several days and subcultures made from day to day. After one week at this temperature, stained specimens from different slants showed forms which could hardly be distinguished from diplococci. These proved on close examination to be minute rods unevenly stained and bipolars. Such cultures, if transferred to serum medium, assumed the typical bacillary form. Figure 18 illustrates clearly how readily one may err in calling these forms cocci. By comparing them with the true cocci shown in Figure 16 the difference can be seen.

Behavior of Coccus and Bacillary Types in Carbohydrates.—Sugar broths (1.0+ to phenolphthalein) were made in the usual manner, and duplicate sets

TABLE 1

ACID PRODUCTION

	C. Enzymicus—Coccus C C	Control C C	C. Enzymicus—Bacillary C C
Dextrose	7.4	1.6	7.0
Lactose	6.1	1.4	7.1
Saccharose	7.2	1.7	6.9
Maltose	8.0	1.3	7.8
Inulin	2.1	1.8	2.0
Dextrin	3.8	1.4	4.4
Glycerol	2.4	1.3	7.4

of tubes were inoculated from a 24-hour old agar culture of C. enzymicus (coccus) and C. enzymicus (bacillary). After 8 days' incubation at 37 C. the titrable acid formed in each of the carbohydrates was determined by addition of N/20 NaOH, using phenolphthalein as an indicator. Titrations were done in the cold. Five c.c. of broth were used in a total volume of 50 c.c. with distilled H_2O.

Examination of the tubes after inoculation with both strains showed the marked difference in rate of growth of the organisms. After 8 hours the coccus type had clouded all of the sugar broths heavily. The bacillary type developed more slowly, and the intensity of clouding after 24 hours approximated that produced by the coccus in a third of the time. Both series showed fine granulation and sedimentation, but this was more pronounced in the case of the coccus.

The acid-production is given in Table 1. The figures represent the amount of N/20 NaOH required to neutralize acid.

The tabulation shows at a glance that, with the exception of glycerol and dextrin perhaps, both types behaved similarly in the carbohydrates used. This might be an argument for similarity or relationship of both organisms. The difference observed in glycerol, however, was too marked to escape notice and

on the basis of this alone would indicate a distinct property not common to both organisms. In order to control the fermentation test, stained preparations were made from both sets of tubes just before titrating, and the apparent similarity of reaction was explained by the selective action of certain mediums on the type of organism. Glycerol was found to inhibit the development of cocci completely; dextrin had a similar action to a lesser degree. In all of the remaining carbohydrates, the bacillary forms had been suppressed to a remarkable extent.

SEROLOGIC EXPERIMENTS

Absorption of Agglutinins.—The object of these experiments was to establish, if possible, by means of highly agglutinating serums, the relationship of the bacillary diphtheroid to the diplococcus. Mellon, in his paper, concludes that the streptococci are related to the diphtheroids and can be derived from these. To establish such a claim it would be necessary to obtain pure strains of each of these two types and show that they are mutually interchangeable. This has not been shown by Mellon, and in the work reported here the results

TABLE 2

ABSORPTION EXPERIMENTS WITH COCCUS SERUM

	Serum C. Enzymicus—Coccus										
	1-20	1-40	1-80	1-160	1-320	1-500	1-1000	1-1500	1-2000	1-3000	1-4000
C. enzymicus (coccus)			±	+	++	++	++	++	++	+	±
C. enzymicus (bacillary)	—	—	—	—	—	—					
After absorption with C. enzymicus (bacillary)											
C. enzymicus (coccus)					++	++	++	++	++	+	±
C. enzymicus (bacillary)	—	—	—	—	—	—					
After absorption with C. enzymicus—Strain 28											
C. enzymicus (coccus)					++	++	++	++	++	+	±
C. enzymicus (28 strain)	—	—	—	—	—	—	—				

do not substantiate his claim. It is evident that two organisms, derived one from the other, should have similar, if not identical, antigenic properties. To test the validity of such an assumption the most delicate method available is that of agglutinin absorption, when the organisms in question are capable of producing agglutinating serums. If the coccus type, obtained from a diphtheroid culture, is truly derived from the bacillus, then a serum prepared with one strain should agglutinate the other, and the absorption of agglutinins should be demonstrable. With this idea in view, two serums were prepared with the coccus strain and the bacillary strain, respectively.

Healthy male rabbits were injected intravenously at 4-6 day intervals with increasing doses of culture heated to 56 C. for 20 minutes. The dosage progressed from $1/20$ of an agar slant to a whole culture. Ten days after the last injection the animals were exsanguinated and the serum, after heating to 53 C. for 15 minutes, was stored at 4 C. until ready for use. The tests were made with serum less than 6 days old.

The technic of absorption was as follows: 24-hour old agar cultures of the organisms were emulsified in a small volume of NaCl solution, heated to 56 C. for 20 minutes, and centrifugated at high speed for a short time. To the sediment so obtained the heterologous serum was added after determining by a preliminary agglutination test the potency of the homologous serum. In order to ensure complete absorption the serums were diluted. The

mixture of bacteria and serum was incubated at 37 C. for 1½ hours, kept at 4 C. over night, and then centrifugated for 20 minutes at high speed. The supernatant serum, pipetted off, was added to another heavy sediment of organisms, obtained as in the preceding, and the absorption repeated, after which agglutination tests were made. A series of dilutions were prepared with each original serum and tubes set up at the same time as the absorbed serum.

The results of these absorption experiments are decisive. A serum prepared with the coccus possesses no agglutinins for the bacillary diphtheroid and when absorbed completely with this bacillary strain evidences no reduction in its agglutination titer against the homologous coccus. The coccus serum when absorbed with a bacillary strain altered morphologically by cultivation at 28 C. for 10 days so that the organism

TABLE 3

ABSORPTION EXPERIMENTS WITH BACILLARY SERUM

	Serum C. Enzymicus—Bacillary									
	1-10	1-20	1-50	1-100	1-200	1-400	1-800	1-1600	1-2000	1-3000
C. enzymicus (bacillary)					++	++	++	+	±	—
C. enzymicus (coccus)	+	—	—	—	—	—				
	After absorption with C. enzymicus (coccus)									
C. enzymicus (bacillary)			++	++	++	++	+	±		
	After absorption with C. enzymicus—Strain 28									
C. enzymicus (bacillary)				±	++	+	—	—		

could not be distinguished from cocci, lost none of its agglutinating power for the coccus. Again evidence is adduced that the change on the part of the bacillus was merely an appearance, and that the organisms were not true cocci, but extremely short bacilli, altered perhaps by changes in surface tension to assume a form remarkably like cocci. Antigenically these organisms are therefore distinct from the coccus type. In Table 3 they are shown to be identical with the original bacillary form. The conclusion to be drawn from this experiment is that the coccus has no antigenic elements in common with the bacillus.

The next experiment, made with a serum produced with the bacillary strain of C. enzymicus, gave additional evidence in another direction and confirmed the difference of the two organisms. The serum, although agglutinating its homologous culture as high as 1-2000, gave agglutination in 1-10 for the coccus. This in itself would be an argument against antigenic relationship. The fact that a bacillary strain is never free from cocci, however, would readily explain slight agglutination which might occur. This interesting point is shown quite clearly

in the absorption test. The serum, after complete absorption with the coccus, showed a reduction in agglutinating titer, stopping at 1-1600, whereas the nonabsorbed serum was positive at 1-2000. This removal of agglutinins might suggest antigenic similarity on the part of the coccus for the bacillus. The important point to be noted here, however, is the cumulative action of multiple doses of bacillary diphtheroids containing cocci in mixture. Repeated injections of mass cultures have resulted in the formation of agglutinins specific for each type of organism, and the slight reduction in titer is thereby accounted for. As a further corollary, Strain 28 absorbs most of the agglutinins out of the bacillary serum, showing that the organisms are antigenically the same. That the absorption is not more complete may be due to the fact that but one exposure was made with the organism in question, whereas in the case of the coccus strain the serum was doubly absorbed. From these findings the conclusion may be drawn that the bacillus is antigenically distinct from the coccus.

Two possibiltics are suggested in explanation of the presence side by side of two biotypes; one is that of mutation and the second a symbiotic relationship of a contaminating coccus and a typical bacillary diphtheroid. The possibility of mutation is not far-fetched, although highly improbable in this instance. As to a symbiosis, the likelihood is less remote. In either case the question can be answered categorically by applying the single bacterial-cell method to the conditions which prevailed in the experiments given here. These experiments will be described in a future paper.

SUMMARY AND CONCLUSIONS

The diphtheroids have been studied with regard to nomenclature and revised according to accepted standards for the naming of valid species.

A tentative classification is offered with a view toward grouping prominent biologic characters. Nine distinct groups of diphtheroids are outlined with a type species for each group and corresponding subspecies.

It is evident that the groups hitherto accepted as types for all diphtheroids are insufficient and not representative of a broad classification.

Diphtheroids isolated from glands and tissues—21 different sources—fall into 12 distinct fermentative groups. The greatest number of organisms are nonfermenters. Strains isolated from the eye and nose

do not attack carbohydrates vigorously, and are usually found either in the nonfermenting or in the dextrose-splitting group.

The diphtheroids associated with Hodgkin's disease have been studied. It has been shown that neither the source nor the cultural characters serve to distinguish the supposed cause of Hodgkin's disease from numerous saprophytic diphtheroids. By means of complement fixation tests the conclusion is reached that the cause of this disease is not the organism described by numerous workers.

Agglutinin absorption studies indicate more clearly the relationship which exists between certain members of the diphtheroid group of bacteria. The results are correlated in the main with complement fixation.

Diphtheroids conform to the fundamental laws of bacteriology and are not readily transformed into cocci and back again. Two distinct biotypes have been isolated from a culture of C. enzymicus.

The coccus associated with a bacillary diphtheroid (C. enzymicus) is antigenically distinct from the bacillus and vice versa. Under certain conditions a bacillary strain may be made to assume a diplococcus picture, but absorption experiments show that the organisms are not cocci.

Certain mediums and cultural technic have been found to exert a definite selection on diphtheroid cultures. One or the other biotype will prevail, depending on the extent of suppression of the first or second.

Two explanations for the existence of coccus and bacillus are suggested, either that of mutation or a symbiotic relationship of a contaminating coccus. The question can be settled by applying the single-cell method to the study.

BIBLIOGRAPHY

Abbot: Bull. Johns Hopkins Hosp., 1891, 2, p. 143.
Baumgarten's Jahrensbericht, 1891, p. 317.
v. Besser cited by Migula.
Biggs, Park and Beebe: Centralbl. f. Bakteriol., 1895, 17, p. 765.
Billings and Rosenow: Jour. Am. Med. Assn., 1913, 61, p. 2122.
Bloomfield: Arch. Int. Med., 1915, 16, p. 197.
Bruno: Berl. klin. Wchnschr., 1898, 35, p. 1127.
Buchanan: Jour. Infect. Dis., 1915, 17, p. 528.
Bunting and Yates: Arch. Int. Med., 1913, 12, p. 236.
Bunting and Yates: Jour. Am. Med. Assn., 1914, 62, p. 516.
Cautley: Rep. Med. Officer of Health, Local Govt. Board, 1894-5, p. 455.
Chester: Manual of Det. Bact., 1901, p. 171.
Demme: Fortschritte d. Medizin, 1888, 1, p. 7.
Eisenberg: Bakt. Diagnostik, 1891.
Enderlen: Ztschr. f. Tiermedezin, 1891, p. 321.

Ernst: Ztschr. f. Hyg. u. Infektionskr., 1889, 5, p. 428.
Ernst: Centralbl. f. Bakteriol., I. O., 1906, 40, p. 79.
Fleming: Lancet, 1909, 1, p. 1035.
Flügge: Ztschr. f. Hyg. u. Infektionskr., 1894, 17, p. 290.
Flügge: Die Mikroorganismen, 1896, 2, p. 477.
Fox: Jour. Med. Research, 1915, 32, p. 309.
Fox: Arch. Int. Med., 1915, 16, p. 465.
Fraenkel: Berl. klin. Wchnschr., 1897, 34, p. 1087.
Fraenkel and Much: Ztschr. f. Hyg. u. Infektionskr., 1910, 67, p. 159.
Galli-Valerio: Centralbl. f. Bakteriol., I. O., 1904, 36, p. 465.
Gerber and Podack: Deutsch. Arch. f. klin. Med., 1895, 54, p. 262.
Gilchrist: Johns Hopkins Hosp. Reports, 1901, 9, p. 409.
Glage: Ztschr. f. Fleisch, u. Milch hygiene, 1903, 13, p. 166.
Goodman: Jour. Infect. Dis., 1908, 5, p. 421.
Graham-Smith: Centralbl. f. Bakteriol., Ref. 1905, 36, p. 358.
Grips: Ztschr. f. Fleisch u. Milch hygiene, 1898, 8, p. 166.
Gromakowsky: Centralbl. f. Bakteriol., 1900, 28, p. 142.
Hall and Stone: Jour. Infect. Dis., 1916. 18, p. 195.
Hamilton: Jour. Infect. Dis., 1907, 4, p. 326.
Hine: Jour. Path. and Bacteriol., 1914, 18, p. 75.
v. Hofmann: Wien. klin. Wchnschr., 1888, 1, p. 65.
Hodara: Monatschr. f. prakt. Dermat., 1894, 18, p. 573.
Jacobson: Annals de l'Inst. Pasteur, 1908, 22, p. 308.
Jacobson: Centralbl. f. Bakteriol., Ref., 1909, 43, p. 213.
Klein: Centralbl. f. Bakteriol., 1900, 28, p. 418.
Kolmer: Jour. Infect. Dis., 1912, 12, p. 56.
Kruse and Pasquale: Ztschr. f. Hyg. u. Infektionskr., 1891, 16, p. 1.
Kunneman: Arch. f. wiss. u. prakt. Tierheilk., 1903, 29, p. 128.
Kurth: Ztschr. f. Hyg. u. Infektionskr., 1898. 28, p. 409.
Landsteiner: Wien. klin. Wchnschr., 1897, 10, p. 439.
Lehman and Neumann: Atlas, 1912, 2, p. 570.
Lesieur: Les Bacilles dit. Pseudo-diphteriques, 1902.
Levy and Fickler: Centralbl. f. Bakteriol., 1901, 30, p. 470.
Lewandowsky: Centralbl. f. Bakteriol., I. O., 1904, 36, p. 473.
Loeffler: Centralbl. f. Bakteriol., 1887, 11, p. 105.
Lubowski: Ztschr. f. Hyg. u. Infektionskr., 1900, 35, p. 87.
Lustgarten: Vierteljahrsschrift f. Derm. u. Syphilis, 1887, p. 914.
Mellon: Jour. Bacteriol., 1917, 2, p. 81.
Migula: System der Bakterien, 2, 1900.
Morse: Jour. Infect. Dis., 1912, 11, p. 251.
Nakanishi: Centralbl. f. Bakteriol., 1900, 27, p. 641.
de Negri and Mieremet: Centralbl. f. Bakteriol., I. O., 1913, 68, p. 292.
Neisser: Ztschr. f. Hyg. u. Infektionskr., 1888, 4, p. 165.
Neisser and Kuschbert: Bresl. ärtztl. Ztschr., 1883, 4.
Nicholas: C. R. de la Soc. de Biol., 1896.
Nicolle: Annals de l'Inst. Pasteur, 1898, 12, p. 186.
Olitzki: Jour. Infect. Dis., 1915, 17, p. 1.
Ortmann: Berl. klin. Wchnschr., 1889, 16.
Plotz: Jour. Infect. Dis., 1915, 17, p. 20.
Preisz and Guinard: Kolle Wassermann Handb. d. path. Mikroorganism, 1913, 5, p. 784.
Prochaska: Ztschr. f. Hyg. u. Infektionskr., 1897, 24, p. 373.
Rhea and Falconer: Arch. Int. Med., 1915, 15, p. 438.

Sabouraud: Annals de l'Inst. Pasteur, 1897, 11, p. 134.
Schwoner: Wien. klin. Wchnschr., 1902, 15, p. 1275.
de Simoni: Centralbl. f. Bakteriol., 1898, 24, p. 294.
de Simoni: Centralbl. f. Bakteriol., 1899, 26, p. 673, 757.
Sternberg: Manual of Bacteriology, 1892.
Südmerson and Thompson: Jour. Path. and Bacteriol., 1910, 14, p. 224.
Torrey: Jour. Med. Research, 1916, 34, p. 65.
Trumpp: Centralbl. f. Bakteriol., 1896, 20, p. 721.
Unna: Monatschr. f. prakt. Dermat., 1891, 15.
Weichselbaum: Beiträge zur. patholog. Anatomie und zur allgem. Pathologie, 4, p. 119.
Zinsser and Hiss: Textbook of Bacteriology, 1915.

EXPLANATION OF PLATES 1-3

Fig. 1.—C. acidum. Large forms developing in mediums enriched with serum. × 1000.

Fig. 2.—Same after prolonged cultivation on ordinary mediums. × 1000.

Fig. 3.—C. suppuratum. On serum medium after 1st isolation. × 1000.

Fig. 4.—Same after prolonged cultivation on ordinary medium. × 1000.

Fig. 5.—C. aurantiacum. × 1000.

Fig. 6.—C. delicatum. × 1000.

Fig. 7.—C. epidermidis. × 1000.

Fig. 8.—C. ascites. × 1000.

Fig. 9.—C. glandulae. × 1000.

Fig. 10.—C. pseudodiphtheriae. × 1000.

Fig. 11.—C. cerebralis. × 1000.

Fig. 12.—C. putidum. × 1000.

Fig. 13.—C. flocculens. × 1000.

Fig. 14.—I. Plain agar slant inoculated with material taken from a culture of C. enzymicus grown on sheep blood glucose agar for 4 generations. The sheep blood agar showed numerous rod forms with cocci intermingled. *A*,[1] heavy white growth superimposed on a delicate transparent background.

II. Plain agar slant inoculated with material taken from opaque, heavy white growth indicated by A^1. *A*, transparent surface growth. *B*, heavy white opaque growth. Smears from A^1 and *B* always showed bacillary forms, typical diphtheroids. *A* yielded pure cocci only. Natural size.

Fig. 15.—Smear from heavy growth indicated by A^1 and *B* in Figure 14.

Fig. 16.—Smear from transparent delicate growth marked *A* in Figure 14. Stained with Loeffler's methylene blue. × 1000.

Fig. 17.—C. enzymicus. Original culture.

Fig. 18.—Bacillary type developing on Loeffler's serum. Transferred from sheep blood agar on which the original culture had been inoculated. When the original strain (Fig. 17) was planted on plain agar, bacilli could not be demonstrated.

Fig. 19.—Suppression of bacillary type on glucose agar. After 3 generations on Loeffler's serum preceded by 2 generations on sheep blood agar, cocci were rare. Note the predominating type here.

Fig. 20.—Transplant from 3rd generation on sheep blood medium to plain agar. The sheep blood culture showed rare cocci and practically a pure culture of bacillary forms.

Fig. 21.—Same culture before transplanting to plain agar. × 1000.

Fig. 22.—C. enzymicus cultivated on blood agar, then transferred to plain agar and incubated at 28 C. "Concentration of chromatin" clearly shown.

Fig. 23.—Transplant from 48-hour veal glucose broth culture of C. enzymicus. Note absence of "transformed" bacilli. The technic according to Mellon.

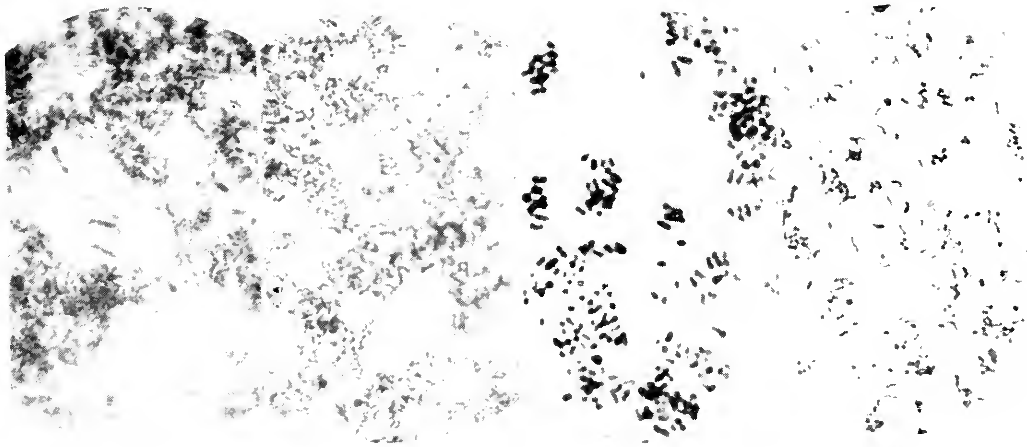
Fig. 24.—C. enzymicus grown at 28 C. in broth. Curious chromatin staining.

Fig. 25.—C. enzymicus 3rd generation on agar from blood medium. This picture, as well as Figure 22, is identical with the "transitional" phase.

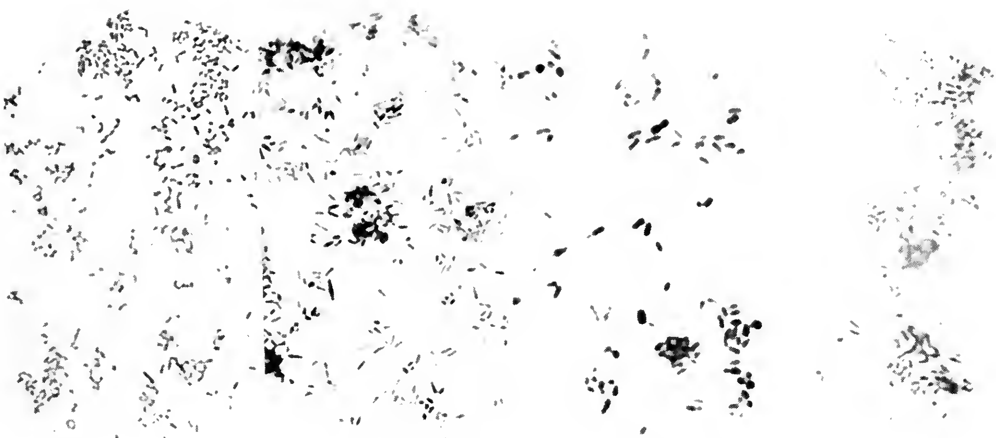
Fig. 26.—C. enzymicus grown at 28 C. on plain agar for 10 days. Note the diplococcus appearance.

Fig. 27.—Previous incubation at 28 C. in broth and subsequent growth at 37 C. Stained according to Kinyoun's method. × 1000.

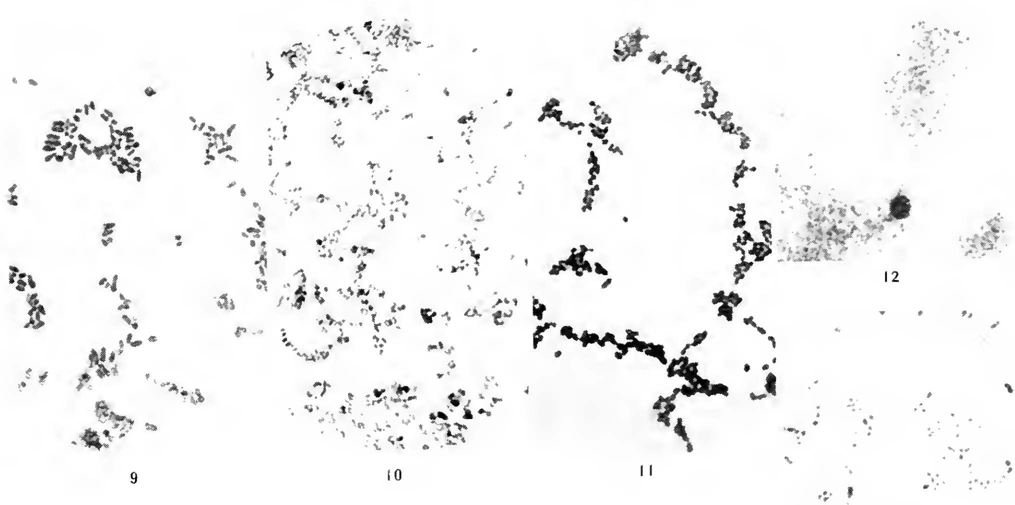
Plate 1



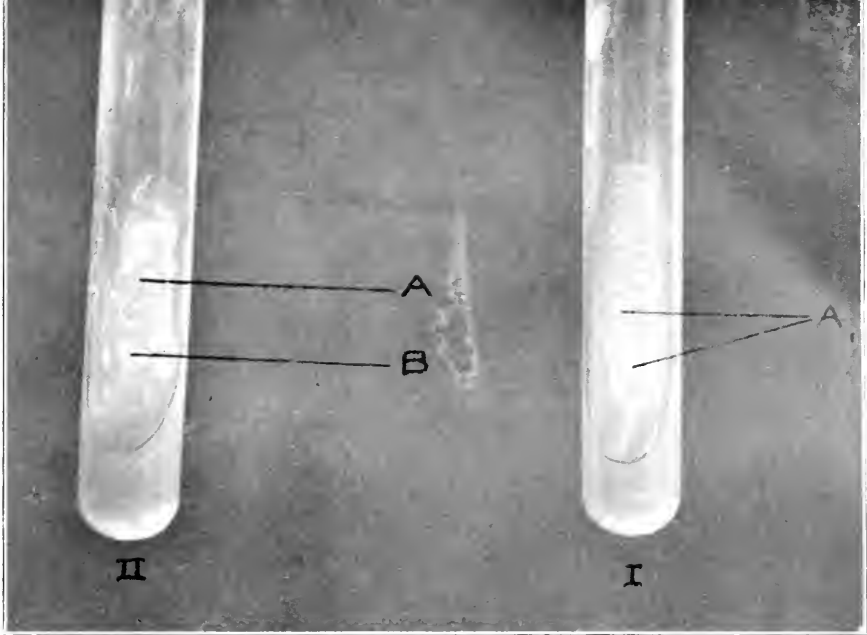
1 2 3 4



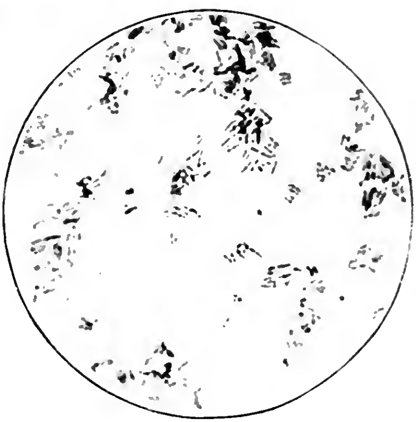
5 6 7 8



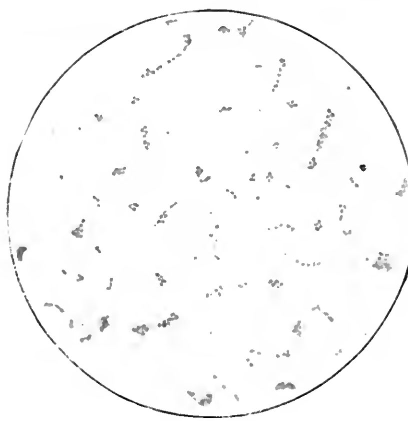
9 10 11 12 13



14

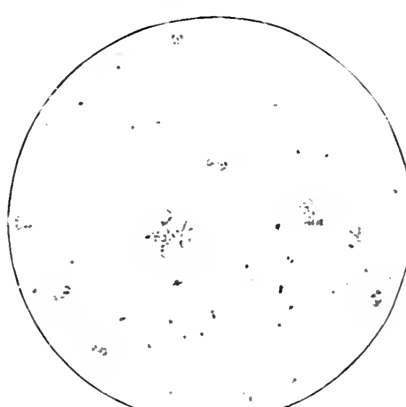


15



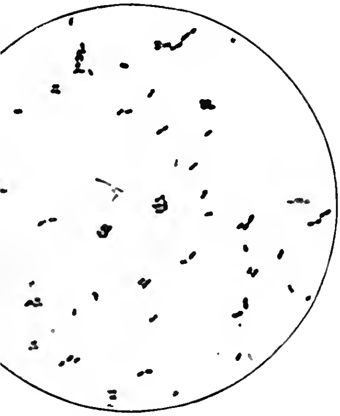
16

17

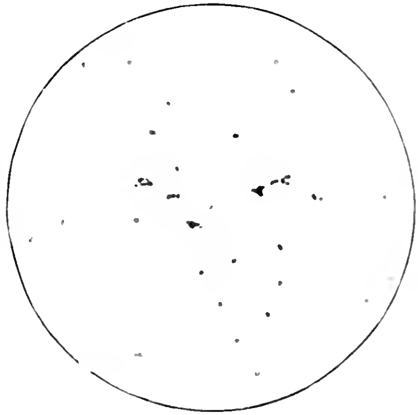


18

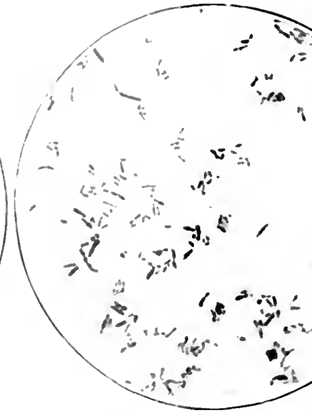
PLATE 3



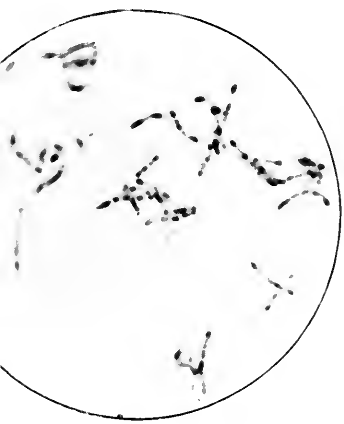
19



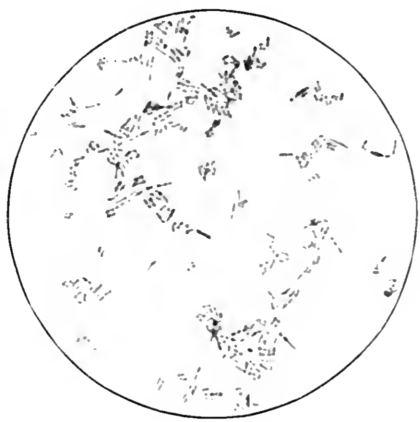
20



21

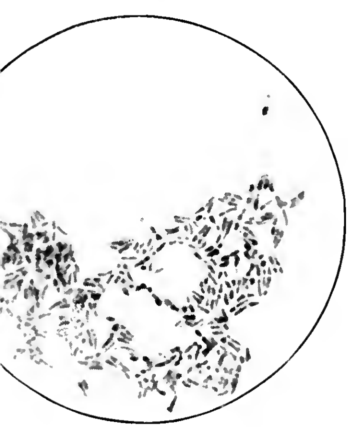


22

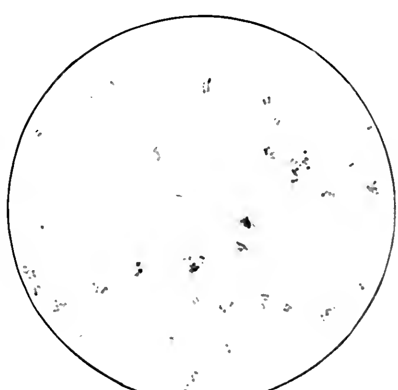


23

24



25



26

27

DIFFERENTIATION OF B. COLI AND B. AEROGENES ON A SIMPLIFIED EOSIN-METHYLENE BLUE AGAR

MAX LEVINE

From the Laboratory of the Department of Pathology and Bacteriology, State University of Iowa, Iowa City, Iowa

For confirming the presumptive test for B. coli the mediums most frequently employed are litmus lactose agar and fuchsin sulphite (Endo) agar. It is becoming more apparent that the coli-like forms may be divided into two groups which are closely correlated with the source. One group (B. coli) is characteristic of fecal origin; the other (B. aerogenes and B. cloacae) is rare in feces, but constitutes the prevailing coli-like form in the soil and on grains. The standard litmus lactose and Endo agar may be employed to a slight extent for the differentiation of B. coli and B. aerogenes, but the differences between these types on these mediums (particularly L.L.A.) are not very clear-cut nor distinct. Better results are obtained with a modified Endo agar described elsewhere. A very excellent differentiation between the B. coli and B. aerogenes types has been obtained on a modification of eosin-methylene blue agar first described by Holt-Harris and Teague for the isolation of the typhoid group from feces. The medium is prepared in the following manner:

Distilled water	1000 c c
Peptone (Difco)	10 gm.
Dipotassium phosphate	2 gm.
Agar	15 gm.

Boil ingredients until dissolved and make up any loss due to evaporation.

Place measured quantities in flasks and sterilize at 15 lbs. for 15 minutes.

Just prior to use add to each 100 c c of the melted agar, prepared as above, the following constituents:

Sterile (20%) lactose solution	1 gm. or 5 c c
Aqueous (2%) eosin (yellowish) solution	2 c c
Aqueous (2%) methylene blue solution	2 c c

Pour medium into petri dishes, allow them to harden in incubator and inoculate in the ordinary way. Smearing the surface with a glass rod seems preferable to the streaking method sometimes employed.

There is no adjustment of reaction and filtration of medium is not necessary.

B. typhosus and members of intermediate group also grow well on this medium producing transparent, colorless, or slightly amber colonies that are about one-half the size of B. coli.

Received for publication Feb. 7, 1918.

DIFFERENTIATION OF B. COLI AND B. AEROGENES ON EOSIN-METHYLENE BLUE AGAR

	B. coli	B. aerogenes.
Size:	Well isolated colonies are 3-4 mm. in diameter.	Well isolated colonies are larger than coli; usually 4-6 mm. in diameter or more.
Confluence:	Neighboring colonies show little tendency to run together.	Neighboring colonies run together quickly.
Elevation:	Colonies slightly raised; surface flat or slightly concave, rarely convex.	Colonies considerably raised and markedly convex; occasionally the center drops precipitately.
Appearance by Transmitted Light:	Dark almost black centers which extend more than ¾ across the diameter of colony; internal structure of central dark portion difficult to discern.	Centers deep brown; not as dark as B. coli, and smaller in proportion to the rest of the colony. Striated internal structure often observed in young colonies.
Appearance by Reflected Light:	Colonies dark, buttonlike, often concentrically ringed with a greenish metallic sheen.	Much lighter than B. coli.. Metallic sheen not observed except occasionally in depressed center when such is present.

RESULTS WITH PURE CULTURES

A number of pure cultures were employed to test the value of this medium for the differentiation of B. coli, B. aerogenes, and members of the typhoid and paratyphoid groups.

Of 22 cultures of B. aerogenes all but 3 gave the characteristic reactions. Of these 3 cultures, 1 resembled B. coli on the eosin-methylene blue agar, another failed to produce a black center, and the 3rd showed a slight metallic lustre, but did not resemble B. coli closely.

Of 35 cultures of B. coli tested, 29 were typical. Six did not show a distinct metallic lustre, but were typical in other respects.

There were 23 strains of B. cholerasuis, B. paratyphosus, and B. typhosus tested. One strain of B. paratyphosus A did not grow. All other strains of the intermediate group developed typical transparent colonies.

RESULTS OBTAINED WITH WATER SAMPLES

The differentiation of pure strains seemed to be so marked and distinct that it was thought the medium might be employed for confirmation of the presumptive test for B. coli, and that it might be pos-

sible to differentiate B. coli from B. aerogenes simultaneously with confirming the presumptive test. For this purpose the following experiment was carried out.

Seven samples of water from different parts of the Iowa river, one of sewage, one of a small creek, and one from a stagnant body of water were plated out directly on litmus lactose agar and inoculated into lactose broth. After 24 hours' incubation 10 acid colonies were fished from the litmus lactose agar plates of each sample for further study. The lactose broth tubes were plated out after 48 hours' incubation onto eosin-methylene blue agar and onto litmus lactose agar. After 24 hours' incubation 10 colonies were fished from these litmus lactose agar plates of each sample for further observation. From the eosin-methylene blue plates made from the preliminary lactose broth tubes colonies which resembled B. coli or B. aerogenes were fished and tentatively designated as such, with a view to determining the accuracy and reliability of the plate differentiation.

All colonies fished from litmus lactose agar were reinoculated into lactose broth and after 24 hours' incubation were plated out on eosin-methylene blue agar. From each plate was picked a well isolated colony which was designated as B. coli or B. aerogenes. These designations were then checked by growing the organisms in Clark and Lubs medium and testing with the methyl-red and Voges-Proskauer reactions.

CULTURES OBTAINED DIRECTLY FROM LITMUS LACTOSE AGAR

Of the 10 water samples examined 1 did not show acid colonies by direct plating on litmus lactose agar. Of the 90 acid colonies fished, 3 were found to be lactose nonfermenters. Thirty-three cultures were regarded, from their appearance on eosin-methylene blue agar, as B. aerogenes. Of these, 24 (72%) gave the Voges-Proskauer reaction. Seven cultures were diagnosed tentatively as questionable but probably B. aerogenes but none of these gave a Voges-Proskauer reaction.

Eight cultures were regarded as questionable, but probably B. coli, of which 6 (75%) did not give the Voges-Proskauer reaction. Thirty-nine cultures were designated from their appearance on eosin-methylene blue agar, as B. coli, and all were confirmed, as none gave the Voges-Proskauer reaction.

Of 40 cultures which were regarded tentatively as B. aerogenes or probably B. aerogenes 24 (60%) were correct. Of 47 cultures regarded as B. coli or probably B. coli, 45 (95.8%) were correct.

CULTURES OBTAINED FROM LITMUS LACTOSE AGAR AFTER PRELIMINARY ENRICHMENT IN LACTOSE BROTH

After elimination of a few strains which proved to be other than coli forms, 85 cultures which were isolated from litmus lactose agar plates made from the lactose broth preliminary enrichment tubes were

smeared onto eosin-methylene blue agar for differentiation. One organism was regarded as probably B. aerogenes and on confirmation proved to be B. coli. Of 35 organisms recorded as B. aerogenes 34 (97%) gave the Voges-Proskauer reaction. Forty-nine organisms were tentatively designated as B. coli or probably B. coli and all proved to be negative for the Voges-Proskauer reaction.

With organisms isolated from this group then, 34 out of 36 cultures regarded as B. aerogenes were confirmed as such while every one of 49 strains regarded as B. coli was correct.

CULTURES OBTAINED FROM EOSIN-METHYLENE BLUE AGAR AFTER PRELIMINARY ENRICHMENT IN LACTOSE BROTH

Fifty-two cultures were fished, 26 of which were regarded as B. aerogenes and the remaining as B. coli. Of the 26 supposedly B. aerogenes strains all gave the Voges-Proskauer reaction. Two of the strains regarded as B. coli also gave the Voges-Proskauer reaction. Thus 100% of the B. aerogenes strains and 92.4% of the B. coli strains were correctly differentiated on eosin-methylene blue agar.

Results on all cultures isolated may be summarized as follows:

Tentatively regarded as B. coli from appearance of eosin-methylene blue agar	122
Correctly designated as indicated by negative Voges-Proskauer reaction	118
Per cent. confirmed	96.9
Tentatively regarded as B. aerogenes from appearance on eosin-methylene blue agar	102
Correctly designated as indicated by positive Voges-Proskauer reaction	84
Per cent. confirmed	82.4

CORRELATION OF VOGES-PROSKAUER AND METHYL-RED REACTION

In previous work a marked correlation was observed between the Voges-Proskauer and methyl-red reactions. Strains of coli-like forms which were acid to methyl-red characteristically did not give a test for acetyl-methyl-carbinal (V-P negative); while those reacting alkaline to methyl-red gave a positive Voges-Proskauer test. These observations were confirmed by Greenfield,[1] Hunter,[2] Clark,[3] Hutton,[4] Rettger and Burton[5] and others.

In this group of strains studied a similar correlation was observed. The relation between the Voges-Proskauer and methyl-red reactions is indicated in the following table:

	Methyl-red		
V.-P.	+	N.*	—
+	2	2	84
—	121	13	2

* In previous work neutral reactions to methyl-red have been grouped with the acid strains.

It is seen from the table that there is an excellent correlation between the two reactions. Placing the neutral reacting strains with the acid group as was done in previous studies, we find that 84 (97.8%) of 86 methyl-red negative strains give the V. P. reaction; while of 136 strains not giving the V. P. reaction 134 (98.7%) were acid or neutral to methyl-red.

In this series of cultures the V. P. reactions were more clear-cut than the methyl-red test but we have worked with collections in which the reverse was true. It seems best to employ both the V. P. and methyl-red tests and to repeat if the results do not agree.

It is interesting to note that of 87 cultures isolated from litmus lactose agar plates made directly, 29.9% proved to be B. aerogenes; whereas of 85 cultures isolated from litmus lactose agar plates made after preliminary enrichment for 48 hours in lactose broth, 40% proved to be B. aerogenes. This indicates the correctness of the contention of Race and others that preliminary enrichment tends to an overgrowth of B. aerogenes types.

Organisms other than B. coli and B. aerogenes grow quite well on this medium and several have been observed to produce small blue centers; but the appearance is so distinct from B. coli and B. aerogenes that once having observed the true types there should be no mistake. Just what these other forms are has not been determined but several have been isolated and will be reported on in a future report. They produce very small colonies with pinpoint light blue or delf-blue centers. The color is very different from the brownish black appearance of the B. coli and B. aerogenes types. Perhaps the introduction of some inhibitory dye into the medium will make it even more reliable for the isolation and differentiation of B. coli and B. aerogenes and confirmation of the presumptive test.

[1] Jour. Infect. Dis., 1916, 19, p. 647.
[2] Jour. Bacteriol., 1917, 2, p. 585.
[3] Jour. Biol. Chem., 1917, 30, p. 209.
[4] Jour. Infect. Dis., 1916, 19, p. 606
[5] Ibid., 21, p. 162.

A COMPARATIVE STUDY OF THE EFFICACY OF THE VARIOUS AGAR-DYE-MEDIUMS RECOMMENDED FOR THE ISOLATION OF TYPHOID AND DYSENTERY BACILLI FROM FECES

K. F. Meyer and J. E. Stickel

From the George William Hooper Foundation for Medical Research, University of California Medical School, San Francisco

Introduction

While investigating peptic and tryptic digests as substrata for culture mediums in routine work, we found that some of these preparations regularly gave better growth of the organisms belonging to the typhoid-dysentery group than the ordinary veal- or beef-infusion or extract-mediums.

This immediately suggested the possibility of improving the existing methods for the detection and isolation of B. typhosus and B. dysenteriae or paradysenteriae. As the result of a few preliminary tests, however, it was quite evident that only a careful comparative study of the existing differential mediums would supply sufficient information to permit of reliable conclusions. The ever increasing reports on elaborate mediums, for the detection of typhoid and dysentery bacilli in stools, show clearly that the problem of this particular technic is not yet solved. And inasmuch as the control of enteric fever and of dysentery will largely depend on the diagnosis and bacteriologic supervision of potential carriers, the knowledge and the application of reliable methods which regularly permit the isolation of the infecting organisms is absolutely necessary even when their numbers are very few. In fact, in experimental work with typhoid carriers in rabbits and other animals, we have encountered difficulties which were traceable to the unreliability of the solid culture mediums used for the detection of typhoid baccilli in the dejecta. The progress of our studies on carriers and dysentery infections depended largely on the development of one or two methods which rendered the isolation of the offending organisms a comparatively simple process.

It is for these reasons that we have retested a large number of recently described methods. In presenting the results, we wish to

* Received for publication Feb. 16, 1918.

point out why negative and misleading findings are frequently obtained by the use of some mediums. At the same time we wish to discuss the advantages of peptic-liver or tryptic beef-heart digests as substrata in the dye-mediums to be applied for the isolation of typhoid and dysentery bacilli. We wish further to demonstrate the relative merits of the various mediums and the immediate purpose for which they are applicable.

Too little attention has been paid to the latter point in connection with the isolation of dysentery bacilli. And, as will be pointed out, the customary assumption that most mediums used for the detection of typhoid bacilli are also suitable for the isolation of dysentery organisms is incorrect. In examining cases of infantile dysentery, in studying the persistence of dysentery bacilli in the feces of convalescence, and in searching for dysentery carriers, we have met with constant disappointment in the results obtained by using Endo's medium (original formula) or litmus lactose agar. Furthermore, certain observations made us suspect that some of the substances needed as indicators in the mediums inhibited or destroyed the bacilli sought for. Later studies have clearly shown that when relatively few bacilli are present, for example, in the formed stools of convalescents, they are frequently not demonstrable on these mediums. The improvements which we suggest are preliminary to investigations which have been in progress for the last two years, and they give promise of producing a selective medium for dysentery bacilli.

For the sake of clearness it appears to be advisable to consider separately our experiments with the various methods and compositions of mediums applicable for the isolation of typhoid and dysentery bacilli.

I. The Detection and Identification of the Organisms of the Typhoid-Paratyphoid Group by Means of Selective Mediums

It has been the general experience that most of the mediums recommended for the detection of typhoid bacilli in stools are more or less inhibitory and that small points of preparation or handling impaired materially the final result. Laboratory workers seem to get the best results usually with the medium to which they are most accustomed. The general use of Endo medium in all the institutes which are handling a great deal of typhoid material is, in our opinion, sufficient proof

of the efficacy of this method. Dreyer, Walker and Gibson[1] as the result of a series of tests of the three differential mediums (MacConkey, Drigalski-Conradi, Endo) in common use concluded in a recent publication that Drigalski-Conradi medium was the least efficient, and Endo decidedly the best. Tidy and Dunn[2] confirmed these observations, but did not find such great differences between the MacConkey and Endo medium. We agree with their statement that, as a rule, more colonies always develop on the ordinary plain agar than on the selective medium. It is unfortunate that Dreyer, Walker and Gibson fail to state the method of preparing the various mediums they submitted to the careful tests which they report. Their results would gain considerably in value if this valuable information had been presented more completely. The MacConkey medium, even though more inhibitive than the Endo medium, has the great advantage of indicating the nonlactose fermenting organisms with striking clearness, which enables the picking out of pure colonies far better than the Endo medium. This particular defect of the latter medium is particularly marked when a soft agar substratum and when a faintly alkaline reaction to litmus is chosen.

Robinson and Rettger[3] tried to overcome the diffusion of the indicator color by recommending a reliable modification of the Endo medium. In our experience their modification is excellent for separating and differentiating typhoid or paratyphoid organisms, when relatively abundant, from a variety of other organisms by selective elimination. But we have observed, as will be shown, that when the typhoid-paratyphoid bacilli are relatively few in numbers, the use of this Endo modification may be misleading as regards the value of a negative finding in a stool examination. We fully agree with Holt-Harris and Teague[4] that the reaction of the Endo medium when adjusted to —0.2 (in our experience a P^H of 7.8-84.4) is too alkaline to permit the optimal growth of the typhoid bacillus, and that this condition limits the usefulness of this type of medium for primary isolation. The modification of Kendall[5] produces less inhibition of growth, but on account of the rapid diffusion of the restored dye through the medium from the B. coli colonies, heavy seeding of the plate is impossible and the picking of colonies is made rather laborious.

[1] Lancet, 1915, 1, p. 643.
[2] Jour. Roy. Army Medical Corps, 1916, 27, p. 482.
[3] Jour. Med. Research, 1916, 34, p. 363.
[4] Jour. Infect. Dis., 1916, 18, p. 600.
[5] Jour. Med. Research, 1911-1912, 25, p. 95.

In recent years these disadvantages have led to the development of selective mediums in which the differentiation of the nonlactose from the lactose fermenting colonies is accomplished by the use of neutral red, or Congo red, or water blue, or china blue as indicators. With the exception of the Congo-red medium of Schmitz,[6] our experiments have shown that these mediums are mostly inferior to the Endo medium. The addition of these dyes to plain lactose agar provokes considerable inhibition of the typhoid bacillus, and does not permit the optimal growth of these bacteria. China blue and water blue in combination with other dyes give, as we intend to demonstrate later, selective mediums of considerable value.

An entirely new method of differentiating nonlactose-fermenting organisms from fecal bacteria has been devised by Holt-Harris and Teague in their eosin-methylene blue agar. Aside from having very little inhibitory action, the medium differentiates typhoid from coli bacilli with most remarkable distinctiveness. No diffusion of the color takes place and the medium can be set to the optimum reaction necessary for the typhoid-paratyphoid bacillus without changing the indicators.

Since Loeffler, and later Conradi, have found that malachite green and brilliant green solutions, respectively, have a marked restraining action on B. coli, numerous solid and liquid mediums have been devised which inhibit the growth of many strains of B. coli and other fecal bacteria to a much greater extent than they inhibit B. typhosus or paratyphosus. Among the many attempts of Lentz and Tietz, Loeffler, Conradi, Fawcus and others to prepare selective mediums with brilliant green or malachite green, only two of the recently described methods, the medium of Krumwiede, Pratt and McWilliams[7] and one of Teague and Clurman[8] deserve further consideration.

The observations of these writers have not as yet been confirmed, and extensive comparative studies with these mediums as regards their practical utility have not been published.

From some of our test experiences we were at once convinced, however, that the eosin-brilliant green medium of Teague and Clurman is the most satisfactory preparation as yet recommended for the isolation of typhoid bacilli from stools. The medium of Krumwiede has certain disadvantages which we shall discuss later.

[6] Deutsch. med. Wchnschr., 1915, 41, p. 426.
Jour. Infec. Dis., 1916, 18, p. 1.
Ibid., 1916, 18, p. 647 and p. 653.

The only objection to the continuous use of Teague's medium in our laboratory, aside, perhaps, from the difficulty in obtaining the proper stains, was the employment of expensive veal infusion peptone agar as a substratum for the dyes. Meat extract preparations also proved unsatisfactory, but the introduction of peptic digests resulted, as already indicated, in the preparation of a cheap, selective medium which surpassed all our expectations. In using such digests the Teague-green medium has been slightly improved and represents in our opinion in this form the only reliable, selectively inhibitive and differentiating medium to be used for typhoid and paratyphoid detection from feces.

After this brief discussion of the status of the methods and their modifications used for the detection of typhoid-paratyphoid bacilli, we desire to present our results under two general captions.

(1) The value of the various indicators, inhibitive dyes, etc., employed in the agar substrata so far as they are outlined in the original descriptions of the methods.

(2) Methods of improving these mediums by use of peptic digests as substrata.

We have[9] called attention to the value of the peptic digests, but in conjunction with our work on the typhoid bacillus the following points deserve particular emphasis.

1. Peptic digests of pig liver and stomach supply amino-acids at an extremely low cost. They furnish complex nitrogenous compounds, which, as Gordon[10] has recently determined, are essential growth factors for the B. typhosus. In this respect they are superior to tryptic digests of beef or casein and the addition of "nutrose" to the extract or infusion agar, which has been recommended by many workers, is superfluous.

2. The dextrose and bile salts in the liver digests are substances which are known to be stimulating to typhoid bacilli. In this connection it should be recalled that Krumwiede as well as Teague[11] have recommended the addition of dextrose to some of their mediums.

3. Digests of blood-clots are equally well suited for typhoid mediums, if one of the processes described in our previous communications is followed. The addition of serum, as Schmitz[12] has suggested, is of

[9] Jour. Infect. Dis., 1918, 22, p. 68.
[10] Jour. Royal Army Med. Corps, 1917, 28, p. 371.
[11] Jour. Med. Research, 1917, 35, p. 107.
[12] Centralbl. f. Bakteriol., I, O., 1915, 76, p. 306.

advantage in connection with such clot digests. In our experience peptic liver digests are less troublesome to make and are therefore to be preferred.

Before giving figures for some of the quantitative experiments, it will be of interest to briefly outline our method of study.

METHODS OF STUDY

The various selective mediums to be tested were carefully prepared according to the original formula, or according to one of the recently recommended modifications. Of the various formulas of special mediums, which we have chosen for quantitative tests, the following are to be mentioned: Endo's medium, original method;[13] modification of Kendall;[5] modification of Robinson and Rettger;[3] Drigalski-Conradi medium;[14] Congo-red agar;[15] malachite-green-china blue agar of Bitter;[16] eosin-brilliant green of Teague;[5] brilliant green agar according to Krumwiede,[7] methylene blue-eosin agar according to Holt-Harris and Teague; MacConkey's medium,[17] and Gonzalez agar.[18]

Particular attention was paid to all details and the various steps given in the descriptions of the methods were faithfully adhered to. Before pouring the plates or before adding the dyes the reaction of the agar medium was determined by one of the colorimetric methods used for the determination of the H-ion concentration. In the experiments with peptic digest substrata, the P_H was adjusted to the same reaction as was determined in previous experiments to be the optimum. This P_H reaction frequently corresponded well with the one of the original method, but differed, for well known reasons, from the titrable reaction. For brilliant green mediums the optimum reaction is—according to numerous tests—P_H 7.0-6.8. Shohl and Janney[19] recently found the maximum growth of B. typhosus (Rawlings) to be at P_H 6.4; our studies showed some variations, the majority of strains grew best, however, at a P_H 7.0-6.4.

All the other peptic digest mediums, with the exception of the Endo's medium, were adjusted to P_H 7.0-7.2. The mediums were poured in constant amounts (25 c c) into large petri-dishes of 12 cm. diameter. As a rule the prepared dishes were dried for 15 minutes in a large incubator (especially installed for this purpose) and used immediately or stored for a few hours. Plates older than 18 hours were not used in these experiments.

Experiments were made by preparing:

(1) Artificial stools.

(2) Known mixtures of pure cultures of B. typhosus and B. coli. High dilutions of these were spread on the surface of the mediums to be tested, and the number of colonies of the two organisms which grew on the plain agar was compared with the number of colonies obtained from the same quantity of the same dilution on the surface of plates of the various special mediums studied.

13 Centralbl. f. Bakteriol., I, O., 1904, 35, p. 109.
14 Ztschr. f. Hyg. u. Infectionskrankh., 1902, 39, p. 283.
15 Deutsch. med. Wchnschr., 1915, 41, p. 427.
16 Centralbl. f. Bakteriol., I, O., 1911, 59, p. 469, and 1913, 71, p. 228.
17 Jour. Hyg., 1908, 8, p. 322, and Thompson-Yates Lab. Rep., 1901, 3, p. 151.
18 Semaine méd., 1913, 32, p. 574.
19 Jour. Urol., 1917, 1, p. 211.

(3) Suspensions of specimens of stool from well known typhoid carriers (rabbits and man) and also from convalescents from typhoid fever or dysentery were spread in known dilutions on the surface of the test mediums.

Numerous experiments with artificial stools did not permit of any quantitative expression of the results, but they confirmed the observations made with stools from carriers, and these are therefore not tabulated.

For the quantitative studies, the method used by Dreyer, Walker and Gibson was followed. The dilutions were prepared from 24-hour old peptic digest broth cultures and the platings were always made with 0.02 c c of the diluted culture or mixture measured by means of accurate, certified serologic pipets. The fluid drop was carefully spread over the surface of the agar by means of a bent 15 gauge "nichrome" wire. The plates were incubated for 24 hours at 37 C.

The preparation of stool suspensions followed the customary procedures; one 2 mg. loopful of the broth or bile suspension was inoculated on each plate and spread by means of the bent wire.

EXPERIMENTAL DATA

Series 1.—In a series of experiments the veal infusion Witte's peptone-agar as a substratum in Endo's, Congo-red, Drigalski-Conradi, eosin-brilliant green and malachite green-china blue medium was compared with peptic digest agar containing the same dyes and indicators. For further comparison, meat extract agar alone and in combination with dyes and indicators, and also the MacConkey medium were included in these tests. The results of three experiments, with three different, recently isolated typhoid and colon strains are summarized in Table 1. The experiments denoted by the same number were made at the same time in each case and were made in duplicate or triplicate.

Table 1 shows clearly that peptic digest mediums are slightly superior to veal infusion agar. These two again give constantly better growth of typhoid bacilli than the ordinary meat extract agar. The differences are not so marked for the colon bacillus.

All the selective mediums containing dyes or indicators inhibit the growth of typhoid bacilli to some degree. This inhibition is characteristic for some mediums like Drigalski-Conradi, Congo-red, and the MacConkey medium; it represents in the viable organisms a reduction of as high as 50%, and it is somewhat more marked on mediums made from veal infusion or on those prepared according to the original formula than on the peptic digest agar preparations. In the Drigalski-Conradi and Congo-red medium the inhibition of the colon bacilli is also striking.

Endo's medium prepared from veal infusion or peptic digests and particularly Kendall's modification show slight inhibition only. Among the brilliant green and malachite green mediums, the Teague medium is distinctly the best. It is of importance to emphasize in this connection that only a few preliminary trials were made to determine the

optimum concentration of the brilliant green in the various batches of agar used; therefore, it is not unlikely that results even better than those shown in Table 1 could have been obtained with Teague medium or with Krumwiede's formula. Considering the data recorded from this viewpoint, the superiority of Teague medium in comparison with the Robinson and Rettger's modification of Endo's medium, is even more striking.

TABLE 1

SHOWING THE AVERAGE NUMBER OF COLONIES WHICH GREW ON VARIOUS MEDIUMS IN EXPERIMENTS MADE WITH VARIOUS KNOWN DILUTIONS OF PURE CULTURES OF B. TYPHOSUS AND B. COLI, RESPECTIVELY

Medium and Indicator	Number of Colonies on Plates Inoculated with B. Typhosus 0.02 C C Experiments			Number of Colonies on Plates Inoculated with B. Coli 0.2 C C Experiments		
	1	2	3	1	2	3
1. Veal infusion, Witte's peptone agar	145	176	55	784	194	25
2. Veal infusion agar, Endo's medium, Robinson-Rettger's modification	112	140	46	680	181	24
3 Veal infusion agar, Congo-red indicator	94	124	48	685	196	20
4. Veal infusion agar, Drigalski-Conradi med.	53	88	—	280	122	—
5. Veal infusion agar, eosin-brilliant green medium of Teague	106	148	53	0	49	4
6. Veal infusion agar, malachite green-china blue medium of Bitter	85	144	—	47	79	—
1. Peptic digest agar	163	296	61	760	204	28
2. Peptic digest agar, Endo's medium, Robinson-Rettger's modification	154	178	47	660	201	25
3. Peptic digest agar, Kendall's modification	—	197	55	—	—	—
4. Peptic digest agar, Congo-red indicator	122	199	49	700	199	21
5. Peptic digest agar, Drigalski-Conradi med.	108	174	—	160	168	—
6. Peptic digest agar, eosin-brilliant green (Teague)	155	225	56	0	94	15
7. Peptic digest agar, malachite green-china blue (Bitter)	120	144	—	323	147	18
8. Peptic digest agar, brilliant green medium of Krumwiede	150	198	53	10	180	14
1. Meat extract (Liebig's) in Witte's peptone agar	105	107	30	725	184	19
2. Meat extract, Endo's medium, original formula	92	90	—	560	142	20
3. Meat extract, brilliant green, Andrade's indicator (Krumwiede)	67	87	24	6	56	4
1. MacConkey's medium	62	75	10	810	200	26

Series 2.—Dilutions of definite mixtures of pure broth cultures of B. typhosus and B. coli were spread on a similar set of mediums as reported in Series 1. The mixtures were used immediately to reduce as much as possible the known antagonistic effect of the colon bacillus on the typhoid bacillus.[20] The observations of Dreyer, Walker and Gibson have already indicated that the typhoid bacillus fails to grow on selective mediums when the proportion of typhoid to colon bacilli in the mixture was lower than 1 typhoid to 15 colon bacilli. We have, therefore, chosen mixtures which were within this range. Table 2 presents two of these tests.

[20] Nissle: Deutsch. med. Wchnschr., 1916, 42, p. 1181.

These experiments confirm the conclusions drawn from the first series and lend considerable support to the observations of Dreyer, Walker and Gibson. MacConkey's medium failed to indicate viable typhoid bacilli when the proportion in the mixtures was 1 typhoid to 15 colon bacilli; in higher proportions also a marked inhibition was noticeable (reduction to nearly 50 per cent.). Among the other mediums the Drigalski-Conradi medium (with crystal-violet) was slightly inhibitive in veal, more so in peptic digest agar. Congo-red, Teague's

TABLE 2

SHOWING THE NUMBER OF COLONIES OF B. TYPHOSUS AND B. COLI, RESPECTIVELY, WHICH GREW FROM MIXTURES MADE AT THE SAME TIME FROM THE SAME CULTURES SO AS TO CONTAIN 15 B. COLI TO EACH B. TYPHOSUS AND 5 B. COLI TO EACH B. TYPHOSUS

Medium and Indicator	Fifteen B. Coli Aerogenes to Each B. Typhosus in Each 0.02 C C		Five B. Coli (Stool) to Each B. Typhosus in Each 0.02 C C	
	Typhoid Colonies	Coli Colonies	Typhoid Colonies	Coli Colonies
1. Veal infusion agar	13	184	230	784
2. Veal infusion agar, eosin-brilliant green (Teague)	6	154	204	0
3. Veal infusion agar, Endo, Robinson and Rettger's modification	7	181	187	680
4. Veal infusion agar, Endo-Kendall's modification	11	126	200	690
5. Veal infusion agar and Congo-red indicator	12	180	180	675
6. Veal infusion agar, Drigalski-Conradi indicator	9	155	103	280
1. Peptic digest agar	17	204	226	760
2. Peptic digest agar and brilliant green-eosin (Teague)	13	200	216	0
3. Veal infusion agar, Endo, Robinson and Rettger's modification	9	224	173	660
4. Peptic digest agar, Endo-Kendall's modification	13	213	174	560
5. Peptic digest agar and Congo-red indicator	14	216	198	610
6. Peptic digest agar, Drigalski-Conradi indicator	4	219	120	160
MacConkey's medium	0	172	125	682

green, and the various modifications of Endo medium gave good results with slight inhibition. The slightly alkaline Endo medium—like Kendall's modification—showed a somewhat higher percentage of viable typhoid bacilli than the strongly alkaline (P_H 7.9-8.4) modification of Robinson and Rettger. Teague's medium proved its superiority only in the test plates which were heavily inoculated with the colon and typhoid bacilli mixture (5:1), probably on account of the ease with which the colonies could be identified and counted. On the other hand,

in the experiments with B. coli aerogenes-B. typhosus mixture, the advantages of the Teague indicator are apparent only in the peptic-digest agar.

Repeated experiments with different mixtures along identical lines, have proven (1) the value of peptic digest agar as a substitute for veal infusion agar, and (2) the excellent quality of the Teague's eosin-brilliant green medium.

Endo and Congo-red medium proved to be equally efficacious in some of these experiments, and as long as light seedings are chosen, they make reliable differential plates. The results with such mediums depend largely, however, on the tedious proper adjustment of the reaction and other factors, which are of minor importance in Teague's medium.

We omitted Krumwiede's brilliant green medium from these experiments because the quantitative results in Series 1 did not show any advantage over Teague's medium; for the purpose of cultural discrimination between B. coli and B. typhosus, Krumwiede's medium contains a less efficacious differential indicator in the decolorized acid fuchsin than the eosin in Teague's medium. Furthermore, the adjustment of the medium to the indicator is delicate, and slight differences are of greater consequence than in the eosin-brilliant green mixture.

Series 3.—To test our observations of Series 2 under the most natural conditions obtainable, we applied the modified Endo's and Teague's medium to our routine feces examination of experimental carriers and of a few human stools. These tests extended over two months and are particularly searching because (1) different lots of digest mediums and indicator solutions were used, and (2) the feces of the rabbit carriers contain (for numerous reasons, to be published shortly) typhoid bacilli, usually in small numbers only. The stool specimens (usually 10 feces balls) were emulsified in sterile ox-bile and incubated for 1 hour, as is customary in our laboratory. One plate of Endo's and one of Teague's medium was each charged with one 2 mg. loopful of the suspension and the drop evenly spread with a bent wire. The following results were obtained:

Total number of carrier stools examined on various days for typhoid bacilli, 472.

Endo medium, Robinson and Rettger's modification, 436 negative, 36, or 7.6% positive.

Brilliant green-eosin medium (Teague and Clurman), 419 negative, 53, or 11.2% positive.

Increased positive results, 3.6%.

Out of 53 typhoid stools only 67.9% were diagnosed by using Endo's medium (R. R. modification).

Proportion of Endo's medium to Teague's medium, 1 : 1.47.

The foregoing tabulation of the stool examinations of carriers shows that it is possible to increase about 30% the positive isolations of the typhoid bacillus by means of the eosin-brilliant green medium. This percentage corresponds well with the one reported by Krumwiede, Pratt and MacWilliams with their brilliant green-acid fuchsin agar.

We fully agree with Teague and Clurman that, in the course of these comparative studies of the two mediums, we have repeatedly found typhoid bacilli on the green plates when the Endo plate gave only questionable colonies, or gave negative results. On the peptic digest-green agar, the typhoid colonies are very large, have a characteristic grayish-pink color and are easily distinguished from the other fecal bacteria which may develop. Aside from the usual roundish colonies, we noted repeatedly a characteristic "mutation type." The colony is large, irregularly shaped, not unlike a grape leaf and shows a network of ridges and furrows in the inside structure. Biochemically and serologically the organisms of these colonies are those of typhoid bacilli, which, as far as our observations have been completed, have not returned to the common type of colony formation inside of 24 generations.

To be sure, some experience is necessary to recognize the typhoid colonies, particularly when numerous B. coli aerogenes strains are present in the specimen of stool. But in our laboratory we have found that even beginners give preference to the Teague's medium for the purpose of differentiating B. typhosus from a variety of intestinal bacteria. Our experience with human stools on Teague's medium is as yet small. We examined daily the feces of six cases of typhoid convalescents by means of the eosin-brilliant green, Endo and malachite green-china blue medium. On several occasions both the Endo and malachite green mediums gave negative results, when the Teague medium revealed several typical colonies. In one series of stool examinations, the Endo medium failed repeatedly to show typhoid colonies even when the other mediums registered a relative abundance of viable B. typhosus. Experiments—to be reported in connection with other studies—demonstrated that the typhoid bacillus responsible for the infection from which the specimens of stool were collected, was only slightly alkali-resistant, in contrast with many other typhoid strains recently isolated. The optimum growth was found to be at an H-ion concentration of P_H 6.2 and multiplication was suppressed at P_H 7.8, which was the reaction of some of the Endo medium samples used for

the tests. This and other observations suggest the use of mediums with a neutral reaction, of which Teague's eosin-brilliant green medium is the one of choice.

Among the many advantages of peptic digests as substrata in Teague's medium, it is well to mention two: (1) the size of the typhoid colonies, and (2) the agglutinability of the typhoid bacillus isolated on such media.

TABLE 3

SHOWING THE NUMBER OF COLONIES WHICH GREW ON THE VARIOUS MEDIUMS IN TESTS MADE WITH VARIOUS KNOWN DILUTIONS OF PURE CULTURE OF B. PARADYSENTERIAE AND B. COLI, RESPECTIVELY

Medium and Indicator	Number of Colonies of B. Paradysenteriae to Each 0.02 C C Experiments		Number of Colonies of B. Coli to Each 0.02 C C Experiments	
	1	2	1	2
1. Veal infusion agar	85	72	509	115
2. Veal infusion agar, methylene blue-eosin, Holt-Harris, and Teague	87 small	68	497	77
3. Veal infusion agar, Endo's medium, Kendall's modification	86	60	501	93
4. Veal infusion agar, eosin-china blue (author's medium)	54	48	482	81
5. Veal infusion litmus lactose agar	58	52	488	82
6. Veal infusion Congo-red agar	68	48	471	72
7. Veal infusion agar, eosin-water blue (author's medium)	55	45	475	94
1. Peptic digest agar	82	98	518	119
2. Peptic digest agar, methylene blue-eosin	88	90	509	96
3. Peptic digest agar, eosin-china blue (author's medium)	67	72	499	88
4. Peptic digest agar, Endo's medium, Kendall's modification	77	58	520	83
5. Peptic digest litmus lactose agar	84	94	480	110
6. Peptic digest Congo-red agar	52	62	460	95
7. Peptic digest agar, eosin-water blue (author's medium)	68	44	478	63
MacConkey's medium	48	85	425	121
1. Meat extract agar	71	—	490	—
2. Endo's medium, modification of Kendall	52	—	468	—

Table 3 shows the average diameter of the various colonies grown on different mediums as determined by means of the microscope and a step micrometer. The figures noted in this table are self explanatory; one large colony supplies sufficient growth for several tentative agglutination tests and the inoculation of several carbohydrate mediums for further identification.

We have been impressed with the relatively small number of inagglutinable strains of B. typhosus which we encountered on Teague's medium, in contrast to the frequent finding of such strains on malachite green and some batches of Endo's medium. These conclusions are reached as the result of several thousands of tentative slides or com-

plete tube-macroscopic agglutination tests. In this connection we found that slightly inagglutinable strains can be made readily agglutinable by conducting the test at a temperature of 50-55 C., or by placing the plate, with the colony to be tested, at this temperature for from 3-4 hours. Using a highly potent goat serum in the dilutions of 1 : 1,000 and 1 : 5,000, together with a control dilution of normal goat serum of 1 : 20, we were able to give a tentative diagnosis in a very short time.

The results obtained by the slide agglutination tests were confirmed by fermentation tests in glucose and in mannite casein digest broth; and by the lead acetate test (P. P. Lévy and Pasteur v. Radot[21] and Kligler[22] in a 0.5% agar made with casein digest broth, by using small tubes, the expense of which is negligible. The additional labor of inoculating three tubes instead of one containing Russell's medium in its original form, or in Krumwiede's and Kohn's[23] modification, is well repaid. From our experience the results obtained on double or triple carbohydrate mediums for fermentation reactions are frequently misleading, because the colonies picked from green mediums are often impure.

FLUID ENRICHMENT MEDIUMS

It is not our purpose to discuss in detail the various fluid mediums which have been recommended for the enrichment of B. typhosus, and which one of us (J. E. Stickel) has investigated. Numerous tests were made with the main object of replacing the peptone solution, or the veal broth used in some of the fluid mediums, by our inexpensive peptic or tryptic digests. We have experimented with the brilliant green enhancement methods of Browning, Gilmour and Mackie,[24] of Robinson and Rettger,[3] of Krumwiede, Pratt and McWilliams,[7] of Tidy and Dunn,[2] and of Teague and Clurman.[4] For the present we feel justified in stating that for rabbit and monkey stools, at least, these mediums have in their original form or modified by the use of peptic or tryptic casein digests, proven unreliable and less efficacious than the direct plating methods on eosin-brilliant green agar. Even by following the advice of Dreyer, Walker and Gibson in using Endo plates, or by diluting the enriched stool culture and plating the dilutions on eosin-brilliant green agar as Teague has suggested, our results were not materially better when natural carrier stools of rabbits or monkeys

[21] Presse méd., Oct. 25, 1915, p. 420.
[22] Am. Jour. Pub. Health, 1917, 7, p. 805.
[23] Jour. Med. Research, 1917, 37, p. 225.
[24] Jour. Hyg., 1913, 13, p. 335.

were used. Artificial human stools frequently showed decided enrichment of the B. typhosus, and more especially when the dye dilutions were used in peptic digests. For rabbit stools we found sterile ox-bile to be an excellent enrichment fluid which, in some recent tests, increased considerably the percentage of positive typhoid findings in the stools. One of us will report on this phase of the problem soon.

The gelatin-Congo-red-brilliant green bromoform enhancement method of Teague and Clurman gave promising results with two human stools. Our experience being based largely on rabbit-carrier stools, is, however, limited and we shall reserve our final conclusions until we have had further opportunities to test with human material. The disadvantages of all the enrichment fluids is that a few more positive findings only are obtained at increased time, cost and labor. We are convinced that, in large comparative series, those apparently favorable results noted with fluid enrichment mediums are counterbalanced by the equally dependable and quicker results obtained by the use of peptic digest-eosin-brilliant green agar according to Teague and Clurman and modified by us.

The method of Morishima and Teague[25] suggested for the isolation of typhoid bacilli from urine has proven satisfactory in our study of experimental renal carriers. The nutrient broth recommended in the original formula can to advantage be replaced by peptic digests.

II. THE DETECTION AND IDENTIFICATION OF THE ORGANISMS OF THE DYSENTERY AND PARADYSENTERY GROUP BY MEANS OF SELECTIVE MEDIUMS

The cultural methods employed for discovering, isolating and identifying the B. dysenteriae present in dejecta are similar to those found useful in examining typhoid patients and for isolating B. typhosus from carriers. Three types of selective mediums have been used as plating substrata, Endo's medium in America, MacConkey's medium in England, and Drigalski-Conradi modified according to Lentz[26] in France and Germany, Denmark (Sonne) and India (Fraser). Recently, Congo-red-agar has been advocated by Lieberman and Acél[27] and Lomas[28] as a more selective medium than litmus lactose agar. As

[25] Jour. Infect. Dis., 1917, 21, p. 145.
[26] Handbuch. d. pathog. Mikroorgan., 1913, 3, p. 924.
[27] Deutsch. med. Wchnschr., 1914.
[28] Boht. Inst. noc. di Higiene de Alfonso XIII, 1915, 11, p. 193, abstracted in Bull. de l'Institut Pasteur, 1916, 14, p. 355.

a rule, primary isolation of the dysentery bacilli does not offer great difficulties because the organisms are usually exceedingly numerous in the mucous flakes of the characteristic dejecta of the acute disease, or during exacerbations. The conditions are entirely different, however, when samples of formed stool of suspected carriers, or stools of children suffering from summer diarrhea, are examined. A high percentage of negative findings is the rule, and only repeated, laborious plating of such specimens will give dependable results. The reports by TenBroeck[29] and others from the Boston Floating Hospital supply sufficient information on this point, so that no further comment is necessary.

One of us[30] (J. E. Stickel) examined, during 1915, fifty cases of infantile diarrhea, using the original Endo method for the isolation of the suspected bacteria with absolutely negative results. During 1916, a similar series of diarrheas was studied with the aid of litmus lactose agar plates, and nearly 80% of the intestinal infections could be diagnosed as "bacillary dysentery." These observations were responsible for the comparative tests which we present in the following paragraph.

The method of study and the character of the experiments were the same as already outlined in the paragraphs on "typhoid." Preliminary tests had shown that the B. paradysenteriae is sensitive to an alkaline reaction of the agar. Mediums were, therefore, chosen in which the indicator reacted best near the litmus neutral point; the optimum H-ion concentration varying between P_H 6.8 to 7.2. It was found that the original Endo formula, Russell's, and Robinson and Rettger's modification were unsuitable and had to be replaced by Kendall's modification. The latter medium does not contain as sensitive an indicator as the other preparations, but is useful in dysentery work.

Veal infusion-peptone and meat extract agar were again compared with peptic digest agar; the outcome of the tests decided in favor of the latter.

Methylene blue-eosin medium of Holt-Harris and Teague[31]—recently recommended for the isolation of typhoid bacilli—gave excellent results when the saccharose was omitted. For the purpose of more striking differentiation we recommend, instead of methylene blue, the use of water or china blue.[32] Only recently, isolated paradysenteriae strains not older than 2 or 3 weeks were used either singly or in mixtures for the study of the various mediums.

[29] Bost. Med. and Surg. Jour., 1915, 173, p. 280.

[30] Master Thesis, University of California, 1916.

[31] Jour. Infect. Dis., 1916, 18, p. 596.

[32] Preparation of Eosin-China Blue or Water Blue Agar: To 100 c.c. of melted peptic liver digest or tryptic beef heart digest agar with a reaction of P_H 6.8 to 7.0, neutral or very slightly alkaline to litmus, add 4 c c of a sterile 20% solution of milk sugar (Merck), heat for 15 minutes in the Arnold and then add 1-2 c c (somewhat less dye is required when American eosin instead of Grübler's or Merck's anilin red is used) of a 2% aqueous solution of yellowish water soluble eosin and 1 c c of a 5% aqueous solution of China blue (Grübler). Shake the mixture very carefully and pour plates. Instead of china blue use 1 c c of a 0.5% aqueous solution of water blue (Grübler) to 100 c c of agar containing lactose and eosin, as already stated above. The dye solutions, prepared with sterile distilled water, should be stored in the refrigerator.

EXPERIMENTAL DATA

Series 1.—In a series of experiments, veal agar as a substratum in methylene blue-eosin, eosin-china blue, eosin-water blue, Congo-red, Endo's medium (Kendall's modification), litmus lactose, and MacConkey's medium, was compared with peptic digest agar media of the same composition. The tests were made with various batches of mediums and were repeated in duplicates. We present in Table 4 the results of two experiments only.

Table 4 shows that peptic digests can advantageously be used for the isolation of dysentery bacilli; in some mixtures of these organisms the development of colonies was increased on the peptic digest mediums (Exper. 2). Furthermore, the size of the colonies is usually twice

TABLE 4

SHOWING THE NUMBER OF COLONIES OF VARIOUS B. PARADYSENTERIAE AND B. COLI WHICH GREW FROM MIXTURES MADE AT THE SAME TIME FROM THE SAME CULTURES

Medium and Indicator	Nine B. Coli to Each B. Paradysenteriae to Each 0.02 C C		Thirty B. Coli to Each B. Paradysenteriae to Each 0.02 C C	
	Dysentery Colonies	Coli Colonies	Dysentery Colonies	Coli Colonies
1. Veal infusion agar	10	55	8	229
2. Veal infusion agar, methylene blue-eosin, Holt-Harris, and Teague	8	44	8	210
3. Veal infusion agar, Endo's medium, Kendall's modification	9	61	4	233
4. Veal infusion agar, eosin-china blue (author's medium)	9	48	5	217
5. Veal infusion litmus lactose agar	5	35	6	210
6. Veal infusion Congo-red agar	12	65	4	224
1. Peptic digest agar	8	45	8	205
2. Peptic digest agar, methylene blue-eosin	8	44	7	222
3. Peptic digest agar, Endo's medium, Kendall's modification	12	42	5	233
4. Peptic digest agar, eosin-china blue	10	46	4	198
5. Peptic digest litmus lactose agar	7	33	5	221
6. Peptic digest Congo-red agar	8	66	4	236
MacConkey's medium	5	42	6	208

the customary one noted on veal agar plates. Table 3 gives detailed information relative to this point. Among the dye indicators the eosin-methylene blue medium showed less inhibition than any of the other preparations tested; the differences, however, are very slight, because most of the mediums chosen are apparently well suited for the isolation of dysentery bacilli. In this connection attention is called to the very good results obtained with MacConkey's medium. This observation is contrary to our previous findings with the typhoid bacillus; even though the colonies are small, differentiation is excellent, and practically all of the viable dysentery bacilli have grown into colonies.

Endo's medium, litmus lactose agar and Congo-red medium are only slightly inhibitive, and can be used advantageously for the differentiation of dysentery bacilli from fecal bacteria.

The addition of china blue or water blue to the eosin-agar of Holt-Harris and Teague produces as a rule a slightly more inhibitive medium than when methylene blue is used. The only advantage these two dyes possess over methylene blue is the power of striking differentiation of the nonlactose fermenting colonies from the lactose fermenting fecal bacteria. Examination of such plates in reflected as well as in transparent light, permits the picking of colorless colonies more readily than on the methylene blue-eosin medium.

TABLE 5

SHOWING THE AVERAGE SIZE OF 20 ISOLATED COLONIES OF B. TYPHOSUS, B. PARATYPHOSUS A, B. PARADYSENTERIAE AND B. COLI ON VARIOUS MEDIUMS AFTER 20-24 HOURS' INCUBATION

Medium and Indicator	B. Paratyphosus	B. Typhosus	B. Paradysenteriae	B. Coli
	mm.	mm.	mm.	mm.
1. Veal agar, eosin-brilliant green	1.8-2.0	1.8-2.2	—	2.8-3.5
2. Veal agar, Endo, Robinson and Bettger's modification	1.6-2.4	1.0-1.5	—	2.2-3.0
3. Veal agar, Endo, Kendall's modification	1.3-1.8	1.3-1.8	1.1-1.4	2.5-2.9
4. Veal agar, Congo-red	1.4	1.3-1.5	1.6-1.8	2.4-3.1
5. Veal agar, Drigalski-Conradi	1.2	1.2	0.8-1.2	2.2-3.3 (No crystal violet)
1. Peptic digest agar, eosin-brilliant green	2.0-2.8	2.1-2.5	—	1.2-1.6 3.2-3.8 (B. coli aerogenes)
2. Peptic digest agar, Endo, Robinson and Rettger's modification	1.3-2.5	0.9-1.4	—	1.8-2.8
3. Peptic digest agar, Endo, Kendall's modification	1.6-2.0	1.6-2.0	2.2-2.8	2.5-3.9
4. Peptic digest Congo-red agar	1.1-1.2	1.3-1.8	2.3-3.8	2.8-4.0
5. Peptic digest agar, eosin-methylene blue	1.8-2.0	1.6-2.5	3.2-4.2	3.2-3.5
6. Peptic digest agar, Drigalski-Conradi	0.9	1.1-1.4	1.1-1.4	2.3-2.7
1. Meat extract brilliant-green agar, Krumwiede	0.4	0.6-1.2	—	1.4-2.0
2. Meat extract, Congo-red	0.8	0.4-1.0	0.8	1.6-2.2
3. Meat extract, Drigalski-Conradi	0.8	0.5-0.8	0.5	1.4
MacConkey's medum	—	0.5-1.0	0.8-1.3	0.8-1.5

Series 2.—A series of dilutions of definite mixtures of pure broth cultures of various strains of B. dysenteriae Shiga and B. paradysenteriae were spread on a set of agar mediums containing various indicators found to be reliable in Series 1. The proportion of the dysentery to the colon bacillus, in the mixtures used, varied between 1 dysentery to 50 colon bacilli. The dysentery bacillus was not found on any of the mediums employed, when present in the mixture, in a proportion less than 1 dysentery to 30 colon bacilli. Varying numbers of B. dysenteriae were recovered from mixtures of 1 dysentery to 5 or 9, 12 or 15, 25 or 30 colon bacilli. The data of two tests are presented in Table 5.

The results shown in Table 5 suggest the following conclusions:

Peptic digest agar with Congo-red indicators, methylene blue-eosin, china blue-eosin, Endo's medium (Kendall's modification), litmus lactose agar and MacConkey's medium, in the order mentioned, are suitable mediums for the isolation and selective differentiation of dysentery bacilli.

We tried to test these conclusions under the most natural conditions, by examining a number of stools of dysentery gall bladder carriers in rabbits. This method of study was unsatisfactory, however, because the infected animals eliminated the dysentery bacilli in large numbers and for short intervals only. All of the mediums compared gave equally good results.

TABLE 6

COMPARATIVE STOOL EXAMINATIONS SHOWING THAT PARADYSENTERY BACILLI INOCULATED FROM SUSPENSION OF FECES ARE INHIBITED ON ENDO'S MEDIUM (ORIGINAL FORMULA)

Patient, Character of Stool and Preparation of Material for Plating	Medium	Total Number of Colonies	Number of Paradysentery Colonies
A. H., liquid, considerable mucus	Peptic digest agar, sugar-free	148	12*
	Peptic digest agar, sugar-free, Endo's original formula	82	5
	Peptic digest agar, sugar-free, methylene blue-eosin (Holt-Harris, and Teague) without saccharose	72	20
	Peptic digest agar, sugar-free, Congo-red (Schmitz)	110	9
	Peptic digest agar, sugar-free, china blue eosin	60	16
	MacConkey's medium	91	14
B. B., watery, diarrheic stool strained through cotton	Peptic digest agar, sugar-free	1,262	98†
	Peptic digest agar, sugar-free, Endo's original formula	1,325	38
	Peptic digest agar, sugar-free, methylene blue-eosin	805	73
	Peptic digest agar, sugar-free, Congo-red	1,185	43
	Peptic digest agar, sugar-free, china blue-eosin	542	61
	MacConkey's medium	1,397	136
M. F., soft but formed stool, convalescent from mild dysentery, suspension shaken and strained through cotton	Peptic digest agar, sugar-free	123	37†
	Peptic digest agar, sugar-free, Endo's original formula	134	8
	Peptic digest agar, sugar-free, methylene blue-eosin	101	56
	Peptic digest agar, sugar-free, Congo-red	128	21
	Peptic digest agar, sugar-free, china blue-eosin	60	32

* Hiss-Y-Russell. † Flexner.

Thus far only three stool samples of paradysentery cases have been available for an actual test of the mediums with which we have experimented. The data collected are presented in Table 6.

The specimens examined came, unfortunately, from acute cases of paradysentery and contained the causative bacilli in abundance. The comparative examinations have therefore only a relative value, but they support our previous observations that the Endo medium (original formula) is inhibitive for the dysentery organisms. Furthermore, Congo-red mediums proved less reliable in these stool examinations than in the previously reported tests with mixtures of pure cultures. This difference is in part due to the heavy seeding of the plates and the diffusion of the acid Congo-red color into the surrounding of the lactose-fermenting colonies. Therefore, preference should be given to the mediums in which the dye is fixed to the colony or diffused only slightly and in which the type of colony can be recognized in reflected light. These requirements are fulfilled by the methylene blue- or china blue-eosin medium which, as these few practical tests indicate, are very slightly inhibitive, or not at all. The advantages of mediums with non-diffusible (methylene blue) or slightly diffusible (china blue) indicators over the other media thus far recommended, have also been noticed on scores of artificial dysentery stools which we have examined.

In addition to the advantages of the methylene blue-eosin agar over the Endo medium already enumerated by Holt-Harris and Teague, we feel justified in adding the following:

1. The expensive meat extract agar can be replaced by a cheap peptic liver digest agar. Sugar free or trypsinized beef heart digests give more striking differentiation of the dysentery colonies than those of the fecal bacteria. For routine work, however, the experienced can use satisfactorily the unfermented digests. On our modification of the methylene blue-eosin medium, the colonies are larger and are therefore more readily recognized.

2. The reaction of the agar does not require careful adjustment, because slight variations fail to affect the efficacy of the indicators. We found a neutral, or very slightly alkaline, reaction to litmus paper or on adjustment to an H-ion concentration of P_H 6.8 to 7.0 the optimum for our digest agar.

3. The plates remain unchanged when exposed to light, and in our experience they fail to deteriorate when kept at ice-chest temperature for over one week. Most of the other mediums, and particularly the Endo medium, are only dependable when fresh.

The use of methylene blue has, in our experience, only one disadvantage, namely, the inhibitive action of this dye on fecal cocci. As a

result of this inhibition, it frequently happens that the colony which is picked from the plate for further identification is not pure. On the other hand, china blue does not inhibit the cocci and the plates prepared with this dye permit of primary isolation of pure colonies.

For the final identification we use carbohydrate solution in sugar-free peptic digests and the agglutination test with polyvalent or monovalent dysentery sera. The detailed procedure will be found in our recent study on paradysentery in California.[33]

Experiments to selectively enrich the dysentery bacilli are still in progress. Unfortunately we have not been able to procure an additional supply of the dyes which have given thus far the most promising results.

CONCLUSIONS

Peptic liver digest agar is a better substratum for the isolation of organisms of the typhoid-dysentery group from stool and urine specimens than veal infusion or meat extract agar.

For the primary isolation of typhoid or paratyphoid organisms from stool and urine of patients or carriers, the eosin-brilliant green medium of Teague and Clurman can be highly recommended, when properly prepared with peptic digests. This solid medium permits the detection of a higher percentage of viable typhoid bacilli than any other one thus far introduced into bacteriologic technic.

Even though our evidence from actual cases is at present small, we consider the eosin-methylene blue medium of Holt-Harris and Teague, or our eosin-china blue medium prepared with peptic or tryptic digests, superior to Endo's medium, litmus lactose or Congo-red agar for the direct isolation and detection of dysentery bacilli from stool specimens.

[33] Meyer and Stickel, Calif. State Jour. Med., 1917, 15, p. 139.

PEPTIC AND TRYPTIC DIGESTION PRODUCTS AS INEXPENSIVE CULTURE MEDIUMS FOR ROUTINE BACTERIOLOGIC WORK

J. E. Stickel and K. F. Meyer

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

In the course of our studies on the carrier problem in enteric infections and our inquiries into the nature and distribution of dysentery infections on the Pacific Coast, we were compelled to investigate the reliability of the various culture mediums which are used. The need of large quantities of mediums made it necessary to find proper or superior substitutes for the reliable, but expensive, veal infusion-peptone-broth. In comparing the merits and values of the numerous vegetable or animal substitutes for "peptone," which have been recommended since the beginning of the war, we found certain peptic and tryptic digests to be excellent substrata for the usual types of culture mediums. These digests are inexpensive, readily prepared in any laboratory, and should therefore be used in conserving the food supply of a country at war.

From the observations of Bainbridge[1] and the studies of Sperry and Rettger,[2] and Rettger, Berman and Sturges,[3] it is evident that the amino-acids and other nitrogenous substances which readily give up their nitrogen as a result of bacterial action, are particularly responsible for the food value of culture mediums. It is a common observation that "Chapoteaut" and certain American peptone solutions give more luxuriant growth of bacteria than an identically prepared solution of Witte's "peptone." Chemical analyses have demonstrated a higher amino-acid content in the "Chapoteaut" (20%) than in American "peptones." Moreover, Duval,[2] Hottinger[5] and others have called attention to the fact that in the usual process of making beef or veal broth the greater part of the nutritive elements of the meat is lost.

Received for publication Feb. 16, 1918.

[1] Jour. Hyg., 1911, 11, p. 341.
[2] Jour. Biol. Chem., 1915, 20, p. 445.
[3] Jour. Bacteriol., 1916, 1, p. 15.
[4] Jour. Med. Research, 1913, 28, p. 165.
[5] Centralbl. f. Bakteriol., I, O., 1912-1913, 67, p. 178.

Indeed, it is not unlikely that in numerous instances very little food material suitable for the pathogenic micro-organisms is extracted when antiquated, uncontrolled methods are used. Therefore, it is not surprising that, as a result of careful comparative studies, Hottinger[5] recommended a process of slow digestion with "pancreatin." Wastefulness is furthermore avoided by this method, as sufficient peptone is obtained by the tryptic digestion to obviate the addition of commercial "peptone." With the exception of the studies of Dalimier and Lancereaux on the value of "opsine" (a digestive product resulting from the combined action of pepsin, trypsin and erepsin on a mixture of proteins) and the recent report of Davis,[7] little attention has been paid in this country to the use of digestion products as culture mediums. Undoubtedly the scarcity of Witte's peptone in England accounted for the investigations of Douglas,[8] Cole and Onslow,[9] which will be considered more carefully in the latter part of this paper.

On the other hand, the failure to obtain strong diphtheria-toxin with peptones other than "Witte's" have prompted several investigators—particularly Robinson and Rettger,[10] and Davis[7]—to ascertain whether the animo-acids, peptones, or some of the various types of proteoses are essential for the development of the toxin. The report of Robinson and Rettger suggested that probably proteoses of polypeptids, resultants of the peptic digestion, are essential to the formation of the toxin. This view is quite in harmony with the fact that Martin's peptone broth and Witte's "peptone" (which as far as present information is available are both peptic-digestion-products) are mediums in which very strong toxins are produced as a rule.

Recent reports by D. J. Lloyd,[11] Cole and Lloyd,[12] and others indicate that highly parasitic micro-organisms, like the meningococcus and gonococcus, require for primary cultivation in vitro certain accessory growth factors (vitamins or growth hormones). The amount of these vitamins necessary to stimulate growth is apparently dependent on the amino-acids present in the medium. From the studies of these workers it is quite evident that the main food requirements of the meningococcus and gonococcus are the products obtained by the hydrolysis of

[6] Arch. de méd. expér. et d'anat. path., 1913, 25, p. 449.
[7] Jour. Lab. and Clin. Med., 1917, 3, p. 75.
[8] Lancet, 1914, 2, p. 891.
[9] Ibid., 1916, 1, p. 9.
[10] Jour. Med. Research, 1917, 134, p. 357.
[11] Jour. Path. and Bacteriol., 1916, 21, p. 113.
[12] Ibid., 1917, 21, p. 267.

proteins. From this brief review it is apparent that the use of peptic or tryptic digestion products is fully justified.

Most of the commercial peptones supply a moderate amino-acid content at a very high cost. On the other hand, tryptic digestion products (of casein, for example) prepared with remarkable ease in any ordinary bacteriologic laboratory, furnish these substances in large quantities and in comparatively constant amounts.

We have, therefore, during the past year studied some of the practical methods recommended for the manufacture of amino-acids and used the same for the preparation of routine mediums. The present report is preliminary to further researches. It is perhaps well to emphasize the point that our main efforts thus far have been concentrated on applying known procedures to utilize animal protein which has either been disregarded as a food source, or has been treated in such a manner that most of the nutritive value has been lost.

The methods employed by us are fully considered in the appendix. The final broth preparations were analyzed chemically[13] to obtain some comparative figures of the most important elements. These data are briefly summarized in Table 1. Only digestion products which repeatedly gave results identical with or superior to the ordinary veal-broth substratum are mentioned. A diversity of micro-organisms, some with very selective food requirements, were tested repeatedly on different lots of digest-mediums.

The following digestion products have thus far been investigated: (1) Tryptic digestion of human placenta. (2) Peptic and tryptic digestion of beef- and sheep-blood. (3) Peptic and tryptic digestion of pig- or beef-liver. (4) Tryptic digestion of beef-heart and "pancreatin medium," according to Hottinger. (5) Tryptic digestion of casein, "tryptamine broth," according to the method of Cole and Onslow. (6) Autolysis of pigs' and dogs' livers, based on the studies of Bradley.

1. *Tryptic Digestion of Human Placenta.*—In bacteriologic laboratories of hospitals the use of placentas as culture mediums can be highly recommended. Simple placental infusions which are used instead of beef or veal are not economical even when they improve the growth of meningococci [(Kutscher)

[13] The determinations were made by means of the following methods:

Total Nitrogen: Kjeldahl's method. Non-Protein Nitrogen: Protein precipitated with trichloracetic acid-Kjeldahl's method on filtrate. Amino-Nitrogen: Van Slyke's method (Jour. Biol. Chem., 1913, 16, p. 121). Chlorids: McLean and Van Slyke's method (Jour. Biol. Chem., 1915, 21, p. 361). Inorganic Phosphates: Marriat's and F. H. Haessler's method (Jour. Biol. Chem., 1917, 32, p. 233).

TABLE 1

RESULTS OF EXPERIMENTS IN THE COMPARATIVE STUDY OF VARIOUS NUTRIENT MEDIUMS

Medium	Total Nitrogen per 1,000 C C	Protein Nitrogen per 1,000 C C	Non-protein Nitrogen per 1,000 C C	Percentage of Protein Nitrogen of Total Nitrogen	Percentage of Amino-nitrogen of Total Nitrogen	Total Solids per 1,000 C C	Ash	Chlorids per 1,000 C C	Phosphates per 1,000 C C	Cost per 5,000 C C of Finished Agar
	Grams	Grams	Grams			Grams	Grams	Grams	Grams	
1. Veal infusion—Witte's peptone broth	3.472	.826	2.646	23.8	10.2-10.6[2]	23.988	4.0885	3.25	0.2	About $1.62[1]
2. Veal infusion—"Difco" peptone broth	3.218	.35	2.898	10.7	13.8	20.924	4.812	....	.....	About $1.74
3. Trypsinized bullock heart according to Douglas	3.08	.956	2.124	31.2	18.5	25.049	3.08	3.32	0.212	76 cents-$1.00 depending on the trypsin used for the process
4. Peptic digest used by the authors:	3.668	....	....	....	14.7	....	....	....	....	About 43 cents-50 cents
a. Lean beef digest......	3.108	....	....	....	......	....	....	....	....	
b. Pig liver digest.......	4.76	1.106	3.654	23.2	17.0-21.0	29.406	7.216	3.75	0.125	24-30 cents[3]
Several samples digested for 24 hours	3.164	....	....	....	......	....	....	....	....	
Several samples digested for 48 hours	3.544	....	....	....	......	....	....	....	....	
Several samples digested for 72 hours	4.620	....	....	....	16.6-17.0	....	....	....	....	
c. Trypsinized pig liver digest 24 hours at 37 C.	3.696	.538	3.158	14.7	35	....	....	....	....	30-33 cents
d. Beef blood clot digest 24 hours	4.13	1.31	2.82	32.2	18	....	....	3.62	0.0930	21-25 cents
5. "Aminoids" (5%) broth....	6.244	....	....	....	28.5	23.08	4.1978	....	....	$1.20
6. Tryptic digest of casein according to Cole and Onslow	3.906	1.143	2.763	29.1	37.0	....	....	....	....	25-40 cents depending on the trypsin used for the process
7. Pancreatin-broth according to Hottinger	7.440	....	....	....	26.0	....	....	....	....	55 cents
8. Whole blood broth according to Szasz and Schmitz	....	....	....	....	......	....	....	....	....	52 cents
9. Liebig's or Lemco extract broth	....	....	....	....	......	....	....	....	....	58 cents
10. Potato broth...............	....	....	....	....	......	....	....	....	....	25 cents

[1] This price includes waste of the veal.
[2] These figures represent averages obtained from several determinations.
[3] This price does not include the expenditure for electric current nor the initial investment for the digester.

or acid fast organisms (Duval and Wellmann)]. Such infusions contain so small amounts of amino-acids (1.5%, Slemons) that they require the addition of commercial "peptones" and mineral elements.

One placenta is sufficient to prepare 2-2.5 liters of broth. Tryptic digestion with ordinary pancreatic extract or trypsin liquor, according to Method 2 of the appendix, gave liquid and solid mediums in every respect similar to the tryptic-broth of Douglas. These cheap digests, when adjusted to a slightly alkaline reaction (P_H 7.3-7.4) and mixed with defibrinated human- or rabbit's-blood, or hydrocele fluid, are particularly suited for the primary isolation of micro-organisms of the respiratory tract and the gram-negative diplococci of the meningo-gonococcus group. Sterilized placental digests keep well when stored at room temperature.

2. *Peptic or Tryptic Digests of Whole Blood or Blood Clots.*—As a substitute for meat, whole blood has been recommended by Szasz,[14] Schmitz[15] and others; their claims have been supported by Kelser[16] who considers the mediums prepared from whole blood superior in many respects to the ordinary laboratory preparations. Our investigations fully confirmed this point, but we were not satisfied with the method of using blood as a substratum, because expensive peptone preparations have to be added to the infusion of the clot and serum. The excellent results noted with the various peptic and tryptic digests of beef-heart or pigs' liver suggested the same procedure for the blood infusion. The methods which proved satisfactory are given under Method III of the appendix.

From Table 1 it will be noted that this blood digest "contains a large percentage of amino-acids" and is exceedingly cheap. The use of this medium in serum institutes, where blood clots are constantly available, can be highly recommended. We have used it as an equivalent substitute for other digests and found it to be excellent for the growth of delicate-growing organisms, like streptococci. In combining the procedure of Schmitz[15] or of Lloyd[11] by adding "vitamines" in form of defibrinated blood to the finished agar, or by clearing the agar with beef serum, a medium resulted which proved of dependable quality for the primary isolation of pneumococci and meningococci. In this form the medium can practically take the place of blood or serum-agar prepared in the ordinary manner.

3. *Peptic and Tryptic Digestion of Pigs' or Beef-Liver.*—Martin's broth, so highly recommended by French workers and others, was proven in our hands the only medium adapted for the isolation of filtrable viruses, particularly the pleuropneumonia virus. Some preliminary tests convinced us that an extraordinary medium for general laboratory work can be procured when, in the preparation of the peptone, there is also included some veal, beef, rabbit muscle, or—as Martin[17] has suggested—some liver pulp. For economic reasons we abandoned the use of beef, but experimented with rabbit muscle which was always available, and we found the resulting medium to be superior to the ordinary veal-broth. Our main interest, however, centered on the applicability of the liver digests. Remarkable results were obtained in our typhoid studies, so that we can fully recommend this digest not only as a substitute for veal-broth or agar, but also as a substratum which gives results superior to those obtained

[14] Centralbl. f. Bakteriol., I, O., 1915, 75, 489 and 77, p. 111.
[15] Ibid., 1916, 76, p. 306.
[16] Jour. Bacteriol., 1916, 1, p. 615.
[17] Compt. rend. Soc. de Biol., 1915, 78, p. 261.

on the usual mediums recommended for the isolation of typhoid and dysentery organisms from the stool. The method of preparing these mediums is fully described in the appendix, Method I.

With the exception of the adjustment of the reaction, no particular difficulties will be encountered in the making of the basic digest broth. Phenolphthalein titration has proven, as usual, to be unreliable, and we recommend either the customary adjustment of the substratum with litmus paper or one of the colorimetric methods used for the determination of the H-ion concentration of biologic fluids. We used the method of Hurwitz, Meyer and Ostenberg[18] in accordance with the suggestions of Clark.[19] The simple method of Cole and Onslow[9] gives equally dependable results. It has been our custom to adjust a total bulk of 10 liters to a reaction of the P_H 7.0, and make the final adjustment in accordance with the special cultural requirements of the organisms for which a batch of medium is used.

A chemical analysis of several different lots of peptic liver digests (25 liters) is shown in Table 1. It will be noted that the percentage of amino-nitrogen, determined by the "Van Slyke method" varies between 17 and 21%. These slight differences are in part due to the filtration of the broth which was not always conducted in the same manner. Similar differences were noted with various lots of veal broth containing the same amount of Witte's peptone from the same powder. In using 5 and more pigs' stomachs and keeping the other factors constant, digests are regularly obtained of a remarkably even composition, the amino-nitrogen representing 18% of the total nitrogen.

In comparison with the veal peptone broth, these mediums contain from four-fifths to twice the amount of amino-acids, as in Witte's peptone broth, together with proteoses which are present in about the same proportion.[20] The amino-acid content of the peptic digest is the same as determined for the trypsinized broth according to Douglas. The total solids and the ash are also increased in comparison with the infusion-peptone-broth.

The only deficiency is found in the phosphate content, which is readily remedied by adding 0.2-0.4% dibasic potassium phosphate (K_2HPO_4) or diammonium phosphate. Henderson[21] and recently Kligler[22] have repeatedly called attention to the extreme value of mixtures of basic and acid phosphate salts in regulating bacterial metabolism and in acting with the proteoses as buffers in culture mediums. A culture fluid, rich in amino-acids but poor in phosphates, will give rapid initial growth which will soon cease, however, on account of the appreciable changes that small increases in acid or alkali have on the H-ion concentration.

Aside from the amino-acid content the presence of carbohydrates is noteworthy. Some samples were fermented with fresh broth cultures of B. coli or of B. saccharolyte; from 10-25% gas was produced inside of 12-16 hours. The presence of these carbohydrates is in part responsible for the excellent growth of the primary cultures of the colon-typhoid group, a point which we shall consider in another paper. For the stock cultures, however, this high carbohydrate content (particularly in the absence of phosphates or similar "buffers")

[18] Bull. Johns Hopkins Hosp., 1916, 27, p. 16.

[19] Jour. Bacteriol., 1917, 2, p. 211.

[20] Two estimations of the proteoses were made by precipitation with saturated zinc sulphate solution.

[21] Jour. Med. Research, 1917, 16, p. 1.

[22] Jour. Bacteriol., 1917, 2, p. 351.

TABLE 2

RESULTS OBTAINED WHEN DEFINITE MIXTURES OF PURE BROTH CULTURES OF B. COLI, B. TYPHOSUS AND B. PARADYSENTERIAE WERE USED

Twenty-Four Hours Old Broth Cultures Plated on:		B. Coli (Stool)			B. Typhosus			B. Paradysenteriae (Hiss-Y-Russell)		
		Million Organisms per C C in an Average of 5 Plates			Million Organisms per C C in an Average of 5 Plates			Million Organisms per C C in an Average of 5 Plates		
		Veal Agar	Peptic Digest Agar	Pancreatin Agar	Veal Agar	Peptic Digest Agar	Pancreatin Agar	Veal Agar	Peptic Digest Agar	Pancreatin Agar
Veal infusion—Witte's peptone broth P_H 7.2	1st Exper.	260	240	210	230	210	260	80	90	120
	2nd Exper.	230	240	240	190	200	180	280	270	250
Peptic digest broth P_H 7.2	1st Exper.	420	450	460	330	360	390	230	250	330
	2nd Exper.	430	160	140	310	350	340	220	210	281
Pancreatin broth P_H 7.2	1st Exper.	170	230	170	190	150	160	40	80	50
	2nd Exper.	150	170	170	180	190	180	50	70	70

TABLE 3

BROTH DILUTIONS OF 1: 1,000,000, 1: 10,000,000 AND 1:100,000,000 PLATED IN 10 C C OF PEPTIC DIGEST AGAR (P_H 7.4)

Organisms Grown in the Following Mediums:	B. Coli			B. Typhosus			B. Paratyphosus			Shiga B. Dysenteriae			(Hiss-Y-Russell) B. Paradysenteriae		
	Million Organisms per C C			Million Organisms per C C			Million Organisms per C C			Million Organisms per C C			Million Organisms per C C		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Veal infusion — Witte's broth P_H 7.4..........	270	>300	450	10	155	205	180	157	192	80	>300	106	170	187	225
Second Exper. { P_H 7.5	333	...	...	243	...	...	...	...	...	...	...	...	...	...	...
Second Exper. { P_H 7.0	231	...	...	138	...	...	...	...	...	...	...	...	...	...	...
Peptic digest broth P_H 7.4....................	280	>300	592	30	263	554	240	183	225	130	>300	180	260	272	211
Second Exper. { P_H 7.5	337	...	...	419	...	...	...	...	...	...	...	...	...	...	...
Second Exper. { P_H 7.0	121	...	...	171	...	...	...	...	...	...	...	...	...	...	...
Pancreatin broth (rabbit) P_H 7.4............	220	470	302	20	70	57	120	125	89	10	4	97	100	73	201

is a disadvantage and should be corrected by the addition of phosphate salts and calcium carbonate. For this purpose it is better, however, to use sugar free digests only.

Most of the broth preparations give a distinct bile salt reaction; in our laboratory digests with strong reactions are particularly reserved for typhoid work.

For the primary isolation of pneumococci, streptococci and like organisms, a higher amino-acid content and known glucose content are sometimes desirable. To accomplish this result, we trypsinized the neutralized sterile digest with 1% trypsin solution for 18-24 hours at 37 C., and simultaneously removed the carbohydrates by adding 0.5% of a 24-hour-old broth culture of B. saccharolyte. Subsequent analyses demonstrated (Table 1) an increase of amino-nitrogen to 35% of the total nitrogen. The final broth contains more suitable protein split products for bacterial growth than the beef broth prepared by slow digestion with "pancreatin" of Hottinger. In comparison with the casein digest prepared according to the method of Cole and Onslow, approximately the same amount of amino-acids is obtained in a considerably shorter time interval.

The progress of the hydrolysis can readily be followed by testing the tryptophane content[23] of the digest in intervals of 12-24 hours. As a rule the peptic digests of liver, blood or beef give only a faint reaction; properly trypsinized mediums, on addition of bromin water, always produce a deep reddish-violet color reaction, the pigment being soluble in amyl alcohol.

According to our experiments, prolonged action of trypsin is inadvisable because the nutritive value is rarely enhanced when the peptic digest is treated for more than 24 hours. The neutralized, trypsinized broth is mixed to advantage with 0.1% dextrose, C.P., 0.2% K_2HPO_4, and 1% calcium carbonate. Agar mediums prepared from the trypsinized liver digest are cleared in our laboratory by the addition of beef or sheep blood (D. Lloyd's "vitamine" agar), and have repeatedly proven to be the most dependable type of culture medium for the isolation of highly parasitic organisms. Our experience of a few months permits us also to conclude that the trypsinized sugar-free liver digest mediums, to which calcium carbonate and phosphate have been added, are well suited for stock cultures of pneumococci, streptococci and meningococci. For various reasons already outlined, peptic digests cannot be recommended for this purpose.

For plating, peptic and tryptic digests can be diluted with tap water from one-half to two-thirds of their volume, or preferably with 0.6% saline or a dilute solution of meat extract. Most of the ordinary micro-organisms grow well on these diluted digests. On the saline diluted agar the number of colonies are frequently increased in comparison with plates made from undiluted digest-or from veal infusion-agar.

The only objection to the regular use of the diluted digests is found in a tendency to the development of small colonies, particularly for our routine work, diluted mediums did not show any advantage over the ordinary standard preparations. Progressive dilution of the digests—as Table 1 readily explains—will reduce the food value of the medium and probably introduce unknown factors with regard to the salt and "buffer" content, which we cannot consider until further studies have been completed.

In Tables 2 and 3 some of the numerous bacteriologic tests, which were carried out with various digests, are enumerated for the purpose of comparing their value with those obtained by the Standard methods. The results are

[23] For quantitative estimation use the method given by Levene and Rouiller, Jour. Biol. Chem., 1907, 2, p. 481.

illustrative of the already stated fact that peptic digests as a rule give a better initial as well as a more prolonged growth of the typhoid-colon group, than "pancreatin media" prepared according to Hottinger or the ordinary veal infusion peptone mediums. The superiority of the peptic digests over "pancreatin media" is conclusively shown in the tables.

We have repeatedly noted that the growth of bacteria on peptic digests is twice as heavy as on the ordinary standard mediums; the observation is particularly valuable in the preparation of bacterial vaccines or large amounts of bacterio-proteins. Peptic liver digests are well suited for indol tests and have also given reliable results as mediums for the detection of gas and acid formation.

Experiments with comparatively small quantities (4-8 liters) of peptic digests demonstrate the possibility of producing a concentrated "extract-like" preparation, which can be handled in a manner similar to Liebig's beef extract. Through the use of heat and a vacuum, concentrated extracts are obtainable, which can be reduced on the water-bath to gummy, brownish, hygroscopic masses. Repeated tests with several small samples proved in every respect identical with the original digest. We are continuing these experiments with the aim of producing peptic digest in easily transportable form, adapted to field and war conditions.

(4) *Tryptic Digestion of Beef Heart* (Douglas) *and the "Pancreatin medium"* (Hottinger).—A series of tests has been carried out with the peptone broth which is obtained by the method of Douglas. This particular medium has attracted considerable attention, being a part of the pea-flour extract medium recommended by Gordon and Flake[24] for the primary isolation of meningococci and by Lloyd for the preparation of stock cultures of these organisms. We can fully support the claims of Douglas: the results obtained with the medium are excellent in every respect. In its original form, or modified for our particular needs, the substratum has a wide range of application. We have used it particularly in Loeffler's serum and egg medium. To encourage the use of this medium, we give in the appendix a detailed outline of the method of preparation.

According to Table 1, the chemical analysis of trypsinized beef heart showed an amino-nitrogen content of 18%, the total nitrogen being somewhat less than are the peptic liver digests. Chlorids and phosphates are present in proper amounts. The great advantage of this tryptic digest over the slow peptic digests consists in the rapidity with which a medium with a high amino-acid content can be prepared. The time to manufacture a good culture medium is considerably shorter than we have found necessary for all the other types of mediums we have investigated. In our opinion, the only objection is the cost. Independent of the price of trypsin, the initial cost of the bullocks' hearts will always double the price of the finished agar in comparison with the peptic liver digest which supplies the same amount of amino-acid by a somewhat more tedious process. On the other hand, beef hearts are less expensive than veal, and we hope that the time is not far distant when the latter will be entirely replaced by the more economical heart muscle.

The "pancreatin medium," made according to the method described by Hottinger, does not offer sufficient advantage over the other mediums, already discussed, to compensate for the trouble involved in its preparation. The "stock-broth," in a dilution of 1 : 10, gives an agar substratum which is inferior to the standard meat-extract-peptone-medium. Many strains of pathogenic bacteria (streptococci, dysentery) on account of the high amino-acid content (Table 1,

[24] Brit. Med. Jour., 1916, 2, p. 678.

7) gave abundant initial growth, but could not be cultivated successfully for any length of time; most of the strains died out after a few transplants. Tables 2 and 3 also illustrate the fact, that "pancreatin" broth or agar is not equal to veal infusion peptone mediums and is inferior to peptic digests of liver or blood.

In substituting rabbit's muscle (which is available in our laboratory from the cadavers of the experimental animals) for beef, we have prepared by the Hottinger process a medium which in a dilution of 1:20 was not only inexpensive but was also of sufficiently constant composition that it could be used by students and beginners in practicing the fundamental bacteriologic technic.

(5) *Casein Digest Prepared According to the Method of Cole and Onslow.*—In previous paragraphs we have referred to the value of tryptic digests. The inexpensive and effective substitute for peptone which was prepared by Cole and Onslow by digesting commercial casein, belongs to this group of digestion products and is therefore included in the scope of our experiments.

We have used a cheap granulated casein which is obtainable in the local market (California creameries) and which is fairly uniform in composition; it is free from carbohydrates and alkalies. Digestion was accomplished with pancreatic extract prepared from pigs' pancreas, or with "Bacto" trypsin.[25] The original method of Cole and Onslow was otherwise carefully followed.

One sample of diluted casein broth, chemically analyzed, gave an amino-nitrogen content of 37%, the highest amount of amino-acid in any finished culture medium thus far studied by us. Other samples, titrated by the Sörensen method and therefore not included in Table 1, gave similar readings. Repeated comparative tests with various organisms, milk and water counts, support the claims of Cole and Onslow that "tryptamine" broth, or agar, supplies at exceedingly low cost a very high concentration of amino-acid in a faintly colored solution which is of fairly uniform composition, provided the same casein product is employed in the process of digestion. In our experience the only disadvantages are the prolonged digestion necessary to obtain a good product and the correspondingly large number of flasks which have to be incubated to insure a regular supply of mediums.

For primary isolation of organisms of the typhoid-dysentery, pneumococci, meningococci group, the peptic liver digests, trypsinized beef heart, or placenta mediums have given better growth than the casein preparations. Some substances, the nature of which is at present unknown, are apparently lacking in the casein digests.[26] Repeated tests have shown that the addition of veal or beef heart infusion (in the proportion of 2 parts infusion to 1 part of casein) give a substratum which is superior to the simple "tryptamine stock broth." Further experiments—already begun by us—are expected to throw some light on these important points and will be published, if they prove to be of any value. For the present we feel justified, however, in recommending the "tryptamine broth" for general application in the study of carbohydrate fermentations and similar bacteriologic procedures (toxin production of spore bearing anaerobes) in which a broth with an abundance of amino-acids, approaching more nearly a synthetic medium, is desirable.

(6) *Liver Autolysates Prepared in Accordance with the Studies of Bradley.*—The recently published studies of Bradley[27] and his collaborators suggested the

[25] Prepared by Digestive Ferments Company, Detroit, Mich., U. S. A.

[26] In this connection we desire to call attention to the observations of Burrows and Neymann (Jour. Exp. Med., 1917, 25, p. 93), that tissue cells are unable to live in the presence of any great concentration of α-amino-acids. It is not unlikely that certain of these acids in high concentrations disturb also profoundly the metabolism of highly organized bacteria.

[27] Jour. Biol. Chem., 1915, 21, 209; 1915, 22, 113; 1916, 25, 261.

use of dogs' or pigs' liver autolysates in N/50 normal hydrochloric acid (1 part of liver to 5 parts of acid) preserved by the addition of chloroform and toluene. After 15-20 days' incubation at 37 C., the deep-brownish autolysates are neutralized with sodium carbonate heated for 30 minutes at 100 C., and filtered. The filtrate diluted in the proportion of 1 part to 20 parts of 0.5% saline. Amino-acid determinations by the Sörensen method gave, on the average, 10-15% amino-nitrogen in the diluted broth. The growth of the test organisms was satisfactory and corresponded well with that obtained with "pancreatin media." Our experiments with these autolysates antedated those with peptic digests and, until quite recently, remained preliminary. The development of a practical method, further detailed chemical analyses, and other important points are still under investigation, and we hope to publish the results in the very near future.

Summary

Inexpensive culture mediums for routine bacteriologic work can be readily prepared from peptic or tryptic digestion products. On account of the high amino-acid content these substrata furnish preparations superior to the usual standard mediums and are of sufficiently constant composition to warrant their general use.

Appendix

I. Preparation of Peptic Digest Broth of Liver, of Beef or of Human Placenta

1. Wash, clean and mince finely 5 or more large pigs' stomachs. Mince an equal amount of clean pigs' or beef liver, cheap fat-free beef, placenta or blood clots.

2. Mix in the following proportions:

Minced stomachs	400 gm.
Minced liver, beef or placenta	400 gm.
Hydrochloric acid (Baker Chemical Co.)	40 gm.
Tapwater at 50 C.	4,000 gm.

and keep the mixture at 50 C. for 18-24 hours.[1]

3. Make a biuret[2] and also a tryptophan test.[3] When both reactions are positive, the digest has a yellowish-greenish color and contains very little undigested debris.

4. Transfer to large bottles[4] and steam for 10 minutes at 100 C. to stop digestion. Strain the digest through cotton, or preferably store over night in the ice-chest and decant after 24 hours.

[1] It is advisable to digest the mixture in glass or porcelain receptacles. We have been accustomed to placing museum jars with the digest in a large, electrically regulated water-bath. Very good results, however, have also been obtained by using a large enameled pot equipped with a "Therm-Elect" thermo-regulator and heating unit installed by the Electric Sales Service Company of San Francisco.

[2] Five c c of filtered digest, add 0.1 c c of 5% solution of copper sulphate; mix and then add 5 c c normal sodium hydroxid. Pink color indicates complete peptonization.

[3] To 10 c c of neutralized and filtered digest add slowly bromin water until the maximum purple coloration is reached.

[4] The bottles in which the Baker Chemical Company supply hydrochloric or sulphuric acid are excellent for this purpose.

5. Warm the filtrate or decanted digest to 70 C. and neutralize with sodium carbonate (twice normal solution) to litmus at this temperature.

6. Sterilize (if not to be used at once) in the autoclave at 10 lbs. pressure for 15 minutes, or for 30 minutes at 100 C., on 2 successive days and store away.[5]

This stock digest is used for the various mediums as follows:

(*a*) *Plain Digest-Broth:*

1. Filter the desired amount into a flask.

2. Add 0.2% dibasic potassium phosphate (K_2HPO_4).

3. Set the desired reaction by using litmus, or preferably to a definite H-ion concentration (P_H 7.0-7.5) by one of the reliable methods recommended for the determination of the H-ion concentration. (Hurwitz, Meyer, Ostenberg, Clark and Lubs, or Cole and Onslow.)

4. Heat the broth in the steamer at 100 C. for 15 minutes.

5. Correct the reaction and filter through paper.

6. Distribute in the receptacles used for cultures and sterilize at 100 C. according to the usual routine method.

(*b*) *Sugar-Free Digest-Broth:*

1. Inoculate the sterile "stock" digest in a flask or bottle with 1% of a 24-hour old broth culture of B. saccharolyte or B. coli and incubate for 12-18 hours at 37 C.

2. Steam for 20 minutes.

3. Adjust the reaction and add 0.2-0.4% dibasic potassium phosphate and 2% purified talcum. Filter through paper, distribute for use, sterilize; or use the turbid, killed saccharolyte culture (without previous cleaning) for the preparation of agar (note method given under "*c*").

(*c*) *Stock Digest Agar:*

1. Take a measured quantity (8-10 liters) of stock digest; add 0.2% dibasic potassium phosphate (K_2HPO_4), and 2% of agar fiber.[6]

2. Autoclave at 10 lbs. pressure for three-quarters of an hour or heat in a double-boiler to 100 C. and keep the mixture at this temperature until the agar is dissolved.

3. Restore the volume lost by evaporation.

4. Set the reaction very lightly alkaline to litmus or to P_H 7.3 by using twice normal NaOH or KOH. Special attention should be given to the adjustment of the reaction, because some commercial agar fiber hydrolyzes readily in the presence of acid.

5. Cool to 60 C., and add the white of egg beaten with the crushed shells (or, for the sake of economy, ordinary beef or sheep serum in the quantity of 25-50 c c per liter[7]).

6. Autoclave for 1 hour at 115 C

7. Filter through cotton and distribute in bottles of 200-500 c c quantities.

8. Sterilize in the customary manner.

[5] Overheating by long continued sterilization should be avoided, the medium becoming dark brown and losing considerable of its nutritive value.

[6] The inexpensive agar fiber of the present market varies considerably in quality and contains from 10-15% water. Some lots of agar will be improved by previous treatment with glacial acetic acid (0.2-0.3%) and subsequent thorough washing.

[7] We are at present engaged in developing a method of clearing agar solution with charcoal. Most of the commercial preparations are very alkaline and repeated adjustment of the reaction makes the process a very tedious one.

(*d*) *Trypsinized Digest Broth:*

1. Peptic digest prepared up to Stage 4 of the general outline is not strained or placed in the ice-chest, but is cooled to 80 C. and made faintly alkaline to litmus with twice normal KOH or twice normal sodium carbonate.

2. Cool to 37 C. and add 1% pancreatic extract (prepared according to the methods given in Plimmer's Practical Organic and Biochemistry, London, 1915, p. 405, or in the paper of Cole and Onslow, *Lancet,* 1916, 2, p. 10) or "Bacto" trypsin (marketed in sterile ampoules by the Digestive Ferment Company, Detroit).

3. Keep the mixture at 37 C. for 3-10 hours depending on the action of the trypsin and the digestion desired. Control the process by repeated tests for tryptophane.

4. When trypsinizing is sufficiently advanced, render slightly acid with glacial acetic acid and bring slowly to boiling point for 10 minutes.

5. Filter through paper, or keep in cool place over night and decant the clear liquid in the morning.

6. Add 0.2% dibasic phosphate, adjust the reaction to faintly alkaline or to the desired H-ion concentration, and treat in the manner outlined in Section "*a*." or use for the preparation of agar.

(*e*) *Sugar-Free Trypsinized Digest Broth:* This can be prepared by the following modification of the method given in Section "*d*."

1. At Stage 2 of the process, add simultaneously with the pancreatic extract or trypsin solution, 0.2% of dibasic potassium phosphate, 1% calcium carbonate and 1% of a 24-hour-old broth culture of B. saccharolyte.

2. Incubate at 37 C. for 12-18 hours and control the digestion by tryptophane tests and the removal of carbohydrates by the gas formation in fermentation tubes.

3. When the digest is sugar-free, steam for 15 minutes and use for the preparation of agar; or

4. Set to the desired reaction and steam for another 15 minutes.

5. Filter through paper; the calcium carbonate present will assist materially in obtaining a perfectly clear filtrate.

6. Distribute and sterilize in the usual manner.

II. Preparation of Tryptic Digest Broth of Human Placenta or Beef Heart

1. Prepare some fresh beef hearts by removing the fat and vessels, mince finely and weigh. Fresh, human placentas are rinsed in water and also passed through a meat-chopper.

2. To 500 gm. of the minced beef hearts or human placentas, add 1,000 gm. of tapwater.[5] Make faintly alkaline to litmus with normal KOH or Na_2CO_3, and heat slowly to 70-80 C. for 5-10 minutes.

3. Cool to 37 C. and add 1% pancreatic extract or "bacto" trypsin (details are given under Heading 1, Section "*d*"), and keep at 37 C. for 2-5 hours. Control the progress of digestion by repeated biuret and tryptophane tests. In

[5] In case distilled water is used or the tap water is poor in calcium and other minerals, calcium, sodium and magnesium chlorid, as well as phosphate, have to be added. The following amounts have proven satisfactory: Sodium chlorid 0.5%; calcium chlorid 0.01%; magnesium sulphate 0.02%, and dibasic calcium phosphate, 0.2%.

case the digestion is extended over a period of 6 hours, it is necessary to add chloroform or toluene.

4. When the process is sufficiently advanced, render slightly acid with glacial acetic acid and boil slowly for 15 minutes.

5. Either filter or decant the clear fluid, which results on placing the digest over night in a cool place.

6. Adjust the reaction, add 0.2% dibasic potassium phosphate and, if necessary, the minerals (chlorid of magnesium, sodium, etc.), in which the broth is deficient for reasons stated in Footnote 8, are added.

7. Heat for 15-30 minutes in the steamer at 100 C. and filter again, if necessary.

8. Sterilize at 100 C. on 3 consecutive days, if not to be used at once.

III. PEPTIC AND TRYPTIC DIGESTS OF WHOLE BLOOD OR BLOOD CLOTS

(*a*) *Peptic Digests:*

1. Obtain from the abattoirs in clean containers 10 liters of fresh beef blood. Decant and store the serum (which has separated on standing) in a refrigerator from 12-18 hours.

2. Weigh the blood clots and mix each 100 gm. with 1 liter of tap water.

3. Place the mixture in an enameled pot, bring slowly to a boil and under constant stirring keep it at this temperature for 5 minutes.

4. Cool to 50 C., add to each liter of the mixture 100 gm. of mixed pigs' stomach (for preparation, see the instructions given in the appendix under Method 1), transfer to glass or porcelain receptacle, and finally add 1% hydrochloric acid.

5. Digest at 50 C. for 18-24 hours and treat the resulting digest as outlined in Method 1. (Steps 3-6, and Section "*a*," steps 1-3).

6. Clear the neutralized broth or agar by adding 5-10% of the decanted beef serum, steam for 45-60 minutes.

7. Remove the flasks or bottles from the steamer and allow the clot to form a compact mass; decant or, better, centrifuge the medium to remove it.

8. Sterilize at 100 C. as customary.

(*b*) *Tryptic Digests:*

1. The preparation of the blood substratum for digestion is practically identical, as given under Section "*a*," Stages 1-4. Use, however, 500 gm. of blood clot to 1 liter of tap water.

2. Strain the fluid portion of the mixture through cheese cloth and pass the residue through a fruit press. Cool to 37 C.

3. Make the thick, brownish fluid slightly alkaline to litmus, add 1% pancreatic extract and keep at 37 C. for 5-24-48 hours.

4. The further treatment of the digest is the same as given under Heading II, Stages 4-8.

5. The neutralized broth or agar can be cleared with decanted serum, the resulting medium is excellent for primary isolation of highly parasitic organisms.

RELATIVE VIABILITY OF B. COLI AND B. AEROGENES TYPES IN WATER

C.-E. A. Winslow and Barnett Cohen

From the Department of Public Health, Yale School of Medicine, New Haven, Conn.

The division of the so-called "colon group" of aerobic gas-forming bacilli into two distinct types, which we owe to the researches of Rogers and his co-workers [1, 3, 6] has opened a wide range of questions, of practical as well as theoretical importance. It seems clear that the B. coli type (low gas ratio, methyl-red positive, Voges-Proskauer negative) is the predominant form in feces while the B. aerogenes type (high gas ratio, methyl-red negative, Voges-Proskauer positive) is apparently the characteristic form found on grains and grasses. The thought has therefore naturally suggested itself that the differentiation between these two types of gas-forming organisms might prove of practical value in sanitary water examinations for distinguishing between surface wash from areas of normal vegetation and those which receive specific fecal pollution. This question of the relative distribution of B. coli and B. aerogenes types in waters of different origin have been discussed by us in another paper.[10]

Aside from this question of the original source of the organisms there is another possibility which might prove significant—the possibility of a difference in the relative viability of the two types in water.

Clemesha,[2] in his very suggestive study of the distribution of colon group organisms in tropical waters, found that in grossly and recently polluted water, B. lactis-aerogenes is a rare organism, but that usually within a period of about 5-15 days after pollution in lakes, this organism becomes extremely common. B. cloacae, another representative of the methyl-red negative type, is one of the most resistant organisms in water. This organism seems to be able to multiply in waters and is the most abundant lactose fermenter in dry weather. Rogers, Clark

Received for publication Jan. 28, 1918.

[1] Rogers, Clark, and Evans: Jour. Infect. Dis., 1914, 15, p. 100. Clark and Lubs: Jour. Infect. Dis., 1915, 17, p. 160.

[2] Bacteriology of Surface Waters in the Tropics, 1912.

[3] Jour. Bacteriol., 1916, 1, p. 82

[6] Jour. Infect. Dis., 1915, 17, p. 137.

and Lubs[3] are of the opinion that B. aerogenes is more resistant to unfavorable conditions than the other more numerous fecal types, and should therefore survive after the latter have disappeared.

We find on study of the data of Houston[4] that of the lactose-fermenting coliform bacteria isolated from 3 types of water the following percentages gave a positive Voges-Proskauer reaction (characteristic of the aerogenes group):

From raw water,	12.9% of 243 cultures
From stored water,	5.3% of 133 cultures
From stored and filtered water,	3.2% of 156 cultures

These figures seem to contradict the idea that the B. aerogenes group is the resistant one. Rogers,[5] on the other hand, in several studies of this question, did find a difference in resistance of the 2 types. Thus, in water artificially infected with feces and held at 20 C there was a gradual change in the ratio of the 2 types until at the end of 9 months there were 39 B. aerogenes to 1 B. coli. In sewage held in running water there was a rapid decrease of colon group bacteria which was more evident with the B. coli type. At the beginning there were about 3 times as many B. coli as B. aerogenes, but after 7 days there were slightly more B. aerogenes than B. coli.

EXPERIMENTAL PROCEDURE

The following experiments were undertaken in order to throw further light on this question of the relative viability of the two types of gas forming organisms.

Ten glass bottles each of 8 liter capacity, and 1 of 16 liters capacity were used. These were all thoroughly washed. Bottles VII to XI were autoclaved prior to filling with water, the others received no further treatment. The bottles were then filled with ordinary tap water to the 7-liter mark—the large bottle to the 14-liter mark. The bottles were stoppered and well shaken, and samples of the water were examined for total count and the presence of gas and acid formers in lactose broth. The counts were always low and gas and acid formers were in no case present.

Each bottle was then inoculated with a loopful of each of 3 different agar slants of B. coli and 3 different cultures of B. aerogenes. The 14-liter bottle, Number VI, received twice the above amount of inoculum. In the case of Bottles I to IV, the loops of culture were shaken directly into the water, and the bottles then thoroughly agitated. In the case of Bottles V to XI, the loops were shaken into 10 c c of dilution water and the resulting suspension

[4] Seventh Research Report to Metr. Water Board, London, 1911.

[5] Bact. Abstr., 1917, 2, p. 56.

thoroughly shaken before being added to the bottles. Promptly after inoculation samples from each bottle were examined in suitable dilutions for

1. Gas production in lactose broth.
2. Acid production on azolitmin-lactose-agar plates.
3. Total count on plain agar plates at 37 C.

Gas production and total count were noted at the end of 48 hours.

The acid colonies were noted from the 12th hour on to the 24th. At the end of 24 hours, 20-25 consecutive acid colonies were picked off and inoculated into the methyl-red medium of Clark and Lubs.[6] The reason for beginning the observation of the plates by the 12th hour was the suspicion that the B. aerogenes type might 'revert' too soon to an alkaline reaction and thus escape detection at the end of 24 hours. Such an early reversion has not, however, been observed very often in this study.

After taking the first set of samples the bottles were set aside in the laboratory and successive samples were taken at regular intervals up to 60 days, the procedure in each test being the same as that described above. The whole work extended from December, 1916, to April, 1917, and the experiments were made in 5 series, begun at different times, 2 bottles being run in each series except the last, which included 3 bottles. Series 1 (Bottles I and II) was stored in a dark closet, the temperature of which varied from about 5 C.-15 C. during the experiment. All the other bottles were stored beneath a table in the laboratory. No attempt was made to exclude diffuse light from these bottles and the temperature varied between 10 and 20 C.

The criterion used for differentiating the B. coli and B. aerogenes types isolated from the stored water was the limiting hydrogen ion concentration attained in the Clark and Lubs medium. This medium, in our experiments, was made up with Difco peptone instead of Witte's. Ordinarily this might introduce some confusion in the test, because some of the organisms of the colon-aerogenes group do not behave alike in mediums made with different peptones. It is suspected that the reason for this difference in behavior lies in the buffer action of the two mediums as well as in the utilizability of the nitrogenous portions.

The particular cultures of B. coli and B. aerogenes used in these tests had all shown a marked stability in the production of a final P_H value in the methyl-red medium made up with Difco peptone. This final P_H was determined by the colorimetric method on 6-8 different occasions for these cultures, at intervals several months apart, and in different lots of the medium. At no time was there any marked variation in the final P_H. The values for the B. coli cultures ran from 4.6-5, while those for the B. aerogenes ran from 6.6-7. For the purposes of this experiment we were therefore safe in using the medium containing the Difco peptone.

The cultures obtained from the stored samples were incubated at 37 C. for 4 days, and then tested with methyl-red solution (0.1 gm. methyl-red dissolved in 300 c c alcohol and diluted to 500 c c with water). B. coli shows a brilliant red coloration while the B. aerogenes culture is yellow.

VIABILITY OF THE GROUP OF GAS FORMERS AS A WHOLE

Turning to our results, we have indicated in Table 1 the total numbers of bacteria per c.c. in the various bottles as tested. Bottles I-IV show a rise in numbers during the first few hours after inocula-

tion. We are somewhat doubtful whether there is any multiplication going on even during this short period. We are rather inclined to attribute the above result to the fact that in the first 2 series the loops of culture were added directly to the water instead of first being thoroughly distributed to break up clumps. In the succeeding series where a preliminary suspension was made in water, we did not find any rise in numbers over the initial count. In any case after this there was a steady decline to the end of the experiments. Bottle V exhibited an unusually fast decline in numbers. On investigation it was discovered that this bottle had for years held bichlorid solution, and apparently, the cleaning it received at our hands failed to remove all traces of the disinfectant.

TABLE 1

Total Bacterial Count in Stored Water (Bacteria per C C)

	Series 1		Series 2		Series 3		Series 4		Series 5			Aver. of All Bottles
Bottles	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	
Time												
0	224,000	161,000	172,000	178,000	125,000	78,000	935,000	877,000	235,000	228,000	225,000	313,000
4 hours	313,000	212,000										
6 hours			179,000	159,000	56,000	64,300	834,000	762,000				
1 day	256,000	194,000	133,000	108,000	20,800	29,700	609,000	610,000	138,000	155,000	180,000	221,000
3			66,700	33,300	1,670	29,000	493,000	466,000	135,000	138,000	132,000	166,000
5	20,700	21,000	2,800	5,600	0	22,000	31,000	36,600	16,000	16,000	24,000	17,800
10	2,700	3,250	990	3,900	0	850	3,000	1,700	4,000	3,300	4,000	2,500
15	1,630	2,600										
20	1,400	1,270	280	1,500		475	2,000	1,600	2,800	1,200	1,200	1,250
30	580	550	180	350		100	200	1,500	1,600	1,100	700	620
40	200	180	100	195		90	140	250	1,400	500	300	305
50	200	100	65	100		80	110	150	150	300	300	141
60	63	67	60	100		60	130	150	70	100	90	81

It is interesting to note that by the 10th day there was a 98 or 99% reduction in numbers in every bottle but one. This point is in agreement with other observations made in experiments comparable with ours. Thus, Houston[7] found a 99% reduction of B. typhi after 1 week of storage in bottles of raw river water. Clemesha also noted a marked diminution in numbers of colon bacilli on storage. Rogers[5] demonstrated a marked falling off in numbers of colon organisms when permeable sacs containing raw sewage were placed in running water for a period of 7 days. Rector and Daube[8] studying the longevity of B. coli in water, noted an initial increase in numbers and then a gradual decrease, with final disappearance of the organisms in about 50 days.

[7] First Research Report to Metr. Water Board, London, 1908.

[8] Bact. Abstr., 1917, 1, p. 57.

RELATIVE VIABILITY OF B. COLI AND B. AEROGENES

In Table 2 are given the percentages of B. coli (positive methyl-red organisms) found at the various periods when the bottles were sampled. It is to be remembered that we aimed at adding approximately equal numbers of B. coli and B. aerogenes to the water in the first place, and the first horizontal line of figures in this table shows how near we came to doing so. This is of course only suggestive, for we realize that when there are several hundred thousand bacteria in a c c it is hard even with all proper precautions to get 20-25 organisms that will be representative of the true relationship between the types present. The results of the various series run closely parallel, however. and we may reasonably conclude that the figures are near the actual truth.

TABLE 2

Per Cent B. Coli Among the Surviving Gas Formers

	Series 1		Series 2		Series 3		Series 4		Series 5			Aver. All Obser-vations
Bottles	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	
Time												
0	50	70	92	92	52	44	32	40	56	24	40	54
4 hours	65	50										
6 hours			92	92	60	40	44	60				
1 day	65	85	88	92	76	80	40	32	56	24	52	63
3 days			96	92	0	60	48	52	40	20	44	50
5 days	45	40	80	80		100	32	36	28	8	32	48
10 days	35	65	76	88		64	32	28	28	8	40	46
15 days	30	55										
20 days	40	65	100	92		48	0	32	12	12	40	44
30 days	15	50	92	92		32	24	28	12	8	40	39
40 days	25	50	92	96		32	16	32	8	0	36	39
50 days	0	40	96	96		20	12	28	8	4	8	31
60 days	5	20	96	100		17	16	28	0	0	4	29

The first point to be noted in these experiments is that there was as a rule an initial increase in the percentage of the methyl-red positive types. This increase reached its maximum during the 1st day in 6 cases. on the 3rd day in 2 cases, on the 5th day in 1 case, on the 20th day in 1 case, and on the 60th day in 1 case.

In all but the last two instances cited (Bottles III and IV of Series 2) there then followed a relative decrease of the methyl-red positive (B. coli) type. In every bottle except the two just mentioned and one other (Bottle VI) the proportion of B. coli was less on the 5th day than at the beginning and after this period the ratio of B. coli to B. aerogenes (in every case except Bottles III and IV) continued to fall off fairly steadily till the end of the experiment.

The behavior of the organisms in Bottles III and IV was anomalous throughout. Instead of a fairly even initial distribution of B. aerogenes and B. coli at the beginning of the experiment these bottles contained in each case 92% of the latter type, which remained predominant to the end of the experiment. The apparent increases to 100% noted on two occasions were merely due to chance inequalities of distribution but the small number of B. aerogenes present did not in any case show an appreciable relative increase.

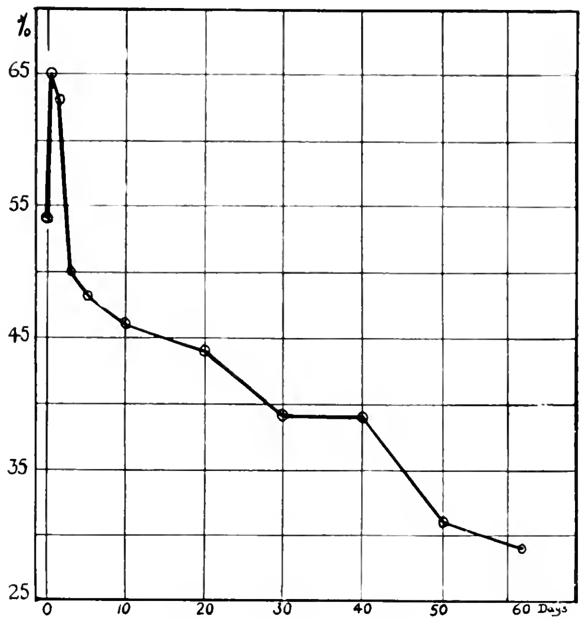


Chart 1.—Ratio of B. coli type to total gas-forming organisms after storage in water for various periods.

In general, however, the lesson of these experiments seems fairly clear. In the last column of Table 2 and in Chart 1 are given the general averages of all observations for each period (except for the periods of 4 hours, 6 hours and 15 days for which there were not sufficient records to be representative). The initial relative increase and marked subsequent decrease of the B. coli type are marked. From the two last columns of Tables 1 and 2 we have calculated the average number of organisms of each of the 2 types present at each period and from these data in turn we have computed the average numbers and

rate of reduction of each type. The results as shown in Table 3 and Chart 2 indicate that neither type followed a normal logarithmic rate of reduction, the rate in each case being very rapid at first and very gradual afterward. This is the sort of death curve which Chick[9] finds to be characteristic of old cultures containing organisms of widely differing vitality. Since the slants from which our bottles were seeded had been incubated for a week this is just what might have been expected.

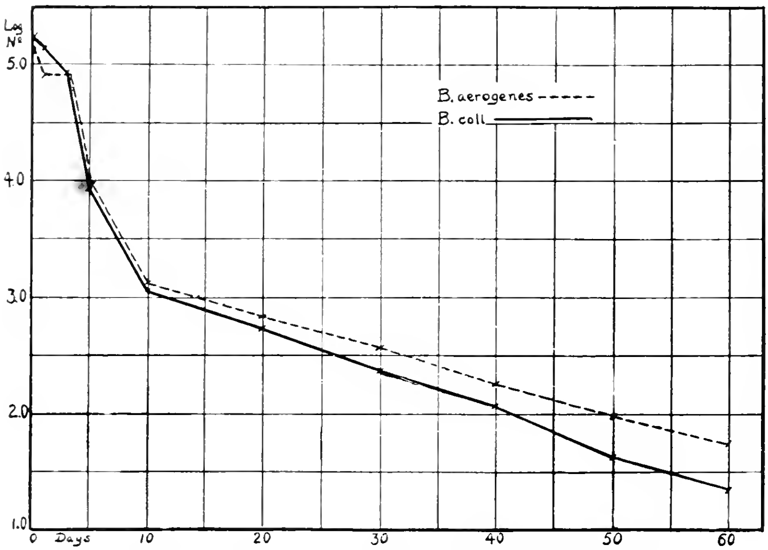


Chart 2.—Reduction of B. coli and B. aerogenes types on storage.

The differences in percentage reductions of the two types were at first quite marked, only 18% of the B. coli having disappeared after 1 day as against 43% of the B. aerogenes. By the 3rd day, however, the reduction of the B. coli was greater than that of the B. aerogenes and after the 5th day the per cent reduction of the 2 types was very nearly alike. If the very sharp drop in both curves at the beginning of storage was due as suggested above to the presence of old cells of low viability the difference between the 2 curves for this first period may have been due to a more rapid multiplication of B. aerogenes in the agar culture used for inoculation and the consequent presence of a larger proportion of susceptible cells.

[9] The Factors Conditioning the Velocity of Disinfection, Orig. Com., Eighth Int. Cong. Applied Chemistry, 26, p. 167.

On the other hand the persistence of B. aerogenes during the latter part of the storage period would appear to be most probably due to an inherently greater viability of this form under the conditions of the experiment. However, the actual differences were very slight as reference to Table 3 will show.

TABLE 3

REDUCTION OF B. COLI AND B. AEROGENES TYPES ON STORAGE

Time	B. Coli			B. Aerogenes		
	No. Present	Log. of No. Present	Reduction per Cent.	No. Present	Log. of No. Present	Reduction per Cent.
0	169,000	5.23	0	144,000	5.16	0
1 day	139,000	5.14	18.0	82,000	4.91	43.1
3 days	83,000	4.92	51.0	83,000	4.92	42.4
5 days	8,700	3.94	94.9	9,100	3.96	93.7
10 days	1,150	3.06	99.3	1,350	3.13	99.1
20 days	550	2.74	99.7	700	2.85	99.6
30 days	242	2.38	99.8	378	2.58	99.7
40 days	119	2.08	99.9	186	2.27	99.9
50 days	44	1.64	99.9	97	1.99	99.9
60 days	23	1.36	99.9	58	1.76	99.9

CONCLUSIONS

In general we may summarize as follows: when a mixture of gas-forming organisms of the B. coli and B. aerogenes types was stored in water under the conditions of these experiments:

All the gas-forming organisms present died off rather rapidly so that in general 98-99% had disappeared by the 10th day.

The B. aerogenes type decreased more rapidly than the B. coli type during the first 24 hours. After that period, however, the B. coli type died off more rapidly than the B. aerogenes type so that while 54% of all gas-forming organisms were of the B. coli type at the beginning of the experiments, this percentage fell to 29 after 60 days.

So far as the results after the 1st day of storage are concerned, our results confirm those of Clemesha and Rogers rather than those of Houston. Studies previously reported[10] indicate that a high proportion of the B. aerogenes type is not, as a matter of actual experience, common even in stored waters. In cases where a high proportion of this sort of gas former is actually found, however, it may apparently be due either to the fact that the gas formers present have come from grains rather than from fecal sources, or to a long period of storage which would tend to increase the relative frequency of the B. aerogenes as compared with the B. coli type.

[10] Winslow and Cohen: Jour. Infect. Dis., 1918,

THE DISTRIBUTION OF B. COLI AND B. AEROGENES TYPES IN POLLUTED AND UNPOLLUTED WATER

C.-E. A. Winslow and Barnett Cohen

From the Department of Public Health, Yale School of Medicine, New Haven, Conn.

In spite of the unique sanitary importance of the colon bacillus and its allies, the classification of this group has, until very recent years, been in an exceedingly unsatisfactory state. It will be remembered, for example, that MacConkey,[1] and Bergey and Deehan,[2] chiefly on the basis of fermentative characters, subdivided the colon family into over a hundred types. The objections that may be raised against such complex and artificial subdivisions are self-evident. MacConkey in the same paper arranged his types of the colon family in four primary groups on the basis of sucrose and dulcite fermentations. Houston divided the colon family into "typical" and "atypical" varieties depending mainly on the fermentation of sucrose. All of these bases for differentiation are purely arbitrary, and they fail to divide the family into naturally occurring species or groups characteristic of any particular habitat.

Far more fundamental has been the work of Rogers and his associates on the gas production and gas ratios of various members of the colon family. By exact methods of gas analysis, they have demonstrated that, in the fermentation of glucose, colon organisms may on the one hand produce gas which is composed of about equal parts of carbon dioxid and hydrogen, or on the other hand may form a mixture made up of two parts of the former to one of the latter component. Later, Clark and Lubs[3] correlated these different gas ratios with corresponding differences in the final hydrogen ion concentration of the glucose broth medium; thus giving a comparatively simple and practical test for differentiating these types. Following on this development came the work of Levine,[4] of Winslow and Kligler[5] and others

Received for publication Jan. 28, 1918.

[1] Jour. Hyg., 1905, 5, p. 333. MacConkey, A.: Jour. Hyg., 1909, 9, p. 86; Papasotirin, J.: Arch. f. Hyg., 1901, 41, p. 204; Prescott, S. C.: Science, N. S., 1902, 15, p. 363; Prescott, S. C.: Medicine, 1903, 11, p. 20; Prescott, S. C.; Biological Studies by the Pupils of William Thompson Sedgwick, 1906, p. 208. Prescott and Winslow: Elements of Water Bacteriology, New York, 1913.

[2] Jour. Med. Research, 1908, 19, p. 175.

[3] Jour. Infect. Dis., 1915, 17, p. 160. Jour. Bacteriol., 1917a, 2, p. 1.

[4] Ibid., 18, p. 358.

[5] Jour. Bacteriol., 1916, 1, p. 81.

which correlated the above characters with the Voges-Proskauer reaction. Thus the colon family may at present be divided into two general groups depending on these three closely correlated characteristics, namely:

(1) B. coli type giving a low gas ratio, positive methyl-red and negative V-P tests.

(2) B. aerogenes type giving a high gas ratio, negative methyl-red and positive V-P tests.

It must, however, be remembered that these two divisions include members whose fermentative powers toward different sugars, alcohols, etc., may be widely unrelated. For instance, we find organisms giving a positive Voges-Proskauer reaction (characteristic of the B. aerogenes group) distributed throughout three of the four primary fermentative groups of MacConkey's classification. The same thing is seen in Kligler's[6] work on the classification of this group. The investigations conducted prior to the introduction of the methyl-red test can therefore be correlated with present information only by means of the Voges-Proskauer reaction.

From a practical sanitary standpoint the important question is whether the normal habitats of the B. coli and B. aerogenes types and their relative distribution in nature are so different as to warrant the placing on their presence in water and food products of a distinct and different interpretation.

Booker,[7] Hammerl,[8] Hellström,[9] and many others report both B. coli and B. lactis-aerogenes (one of the commonest representatives of the B. aerogenes group) as present in human stools. MacConkey[1] was unable to isolate by the enrichment method any of the B. lactis-aerogenes group from a normal specimen of feces—the 36 cultures isolated were all V-P negative. He examined other specimens with the special purpose of isolating B. lactis-aerogenes, but in these he did not use the V-P test, presumably depending on sugar fermentations for identification. He found only 4 out of 241 strains from human feces to be definitely of the fermentative B. lactis-aerogenes type. He found no B. cloacae present. He was also struck by the absence of B. lactis-aerogenes in a series of 99 cultures isolated from horse, cow, and calf feces.

Ferreira, Horta and Paredes[10] studied 117 strains of lactose fermenting bacilli from human feces. The 'Proskauer reaction' (apparently the Voges-Proskauer reaction, though it is not quite clearly stated) was positive in glucose broth only 8 times out of the 117 cultures. This would indicate that the B. aerogenes group was represented in their collection to the extent of 6.8%. MacConkey

[6] Jour. Infect. Dis., 1914, 15, p. 187.

[7] Centralbl. f. Bakteriol., 1891, 10, p. 284. Bowles, J. T. B.: Am. Jour. Pub. Health, 1916, 6, p. 1173.

[8] Centralbl. f. Bakteriol., 1897, 1, Ref. 22, p. 706.

[9] Ibid., 30, p. 309.

(1909) in a further study found 11 cultures, or 6.2% of 178 strains isolated from human feces to give a positive Voges-Proskauer reaction. In horse feces he found 8 cultures, or 11.9% of 67 strains to be Voges-Proskauer positive. In 87 strains from calf, goat, pig, and goose feces he records no Voges-Proskauer positive cultures. In addition, he reports 68, or 56.2% Voges-Proskauer positive strains out of a total of 121 bacilli isolated from corn ear, malt, beans, oats, cheese, rain water, roof washings, pond water, and soils, the latter representing different degrees of possible contamination. Houston[11] gives a series of data on cultures isolated from waters. Among the tests are included lactose fermentation and the V-P reaction. From these we have calculated the percntage of lactose fermenters in his series which gave a positive V-P reaction, and find that about 13% of the strains from raw water, 5% of those from stored water, and 3% of those from filtered water were of this type. Clemesha[12] made an extended study of the colilike organisms in human and bovine feces. Nine series were run with the human feces through a period of 3 years; 104 samples of feces were examined, and the results are based on 1,207 cultures isolated. There were 10 series of cow feces through the same period, comprising 86 samples and 1,029 cultures isolated therefrom. The average percentages of the Voges-Proskauer positive organisms recorded as compiled from his results were 6.06% for the human and 10.66% for the bovine strains.

The next series of extended researches is that of Rogers and his co-workers. In 1914 Rogers, Clark and Evans[13] found but one out of 150 strains isolated from bovine feces to be of the high ratio, B. aerogenes, type. Later,[14] the same authors, in a study of 166 organisms isolated from grains found 151 to be of the high ratio, B. aerogenes type. They note that the types found on grains are characterized by pigment formation on agar. Rogers, Clark and Lubs[15] in a study of the colon bacteria in human feces found 107 out of 113 to be of the low ratio, B. coli, type, the remaining 6 being of the type corresponding to the predominating group in the grain cultures reported above. They conclude, however, that "while this (grain) type occurred in relatively small numbers (in feces), the actual number may amount to several hundred thousand in each gram of material. It is possible that the more frequent occurrence outside of the animal body of the high ratio type may be because it is more resistant to unfavorable conditions and consequently survives after the low ratio type disappears." The same authors in a more recent paper (unpublished manuscript, 1917) include 63 more cultures of which 40 were high ratio cultures. Out of the total of 176 strains 46, or 26%, were therefore of the B. aerogenes type. Rogers[16] examined the types of colon bacilli occurring in surface waters, and found out of 137 strains isolated, about one-third of the low ratio, B. coli, type. This form was found occasionally in springs in which there was no evident source of contamination, but was especially abundant in streams polluted with sewage. He states that the significance of the presence of this type in water cannot be determined.

Levine[17] in a study of 187 cultures from sewage and feces found that only 28 gave a positive V-P reaction. He states that the colilike bacteria which give this reaction are characteristically of nonfecal origin, that they resemble B.

[10] Arc. D. R. Inst. Bact. Camara Pestana, 1908. Tome II, Fasc. II, p. 153.

[11] Seventh Research Report to Metr. Water Board, London, 1911.

[12] The Bacteriology of Surface Waters in the Tropics, 1912.

[13] Jour. Infect. Dis., 1914, 15, p. 100.

[14] Rogers, Clark and Evans: Jour. Infect. Dis., 1915, 17, p. 137.

[15] Jour. Bacteriol., 1916, 1, p. 82. Unpublished manuscript, 1917.

[16] Ibid., p. 82.

[17] Ibid., p. 87.

aerogenes (Escherich) and probably represent soil forms. In another paper[18] this author states: "The Voges-Proskauer reaction is of considerable sanitary significance. It differentiates between fecal and nonfecal colilike organisms and may be an index of soil washings." In still another communication[19] he reports that the methyl-red negative, V-P positive organisms were not uncommon in sewage, but rarely present in the feces of man, horse, sheep, pig, and cow. Johnson[20] in an examination of 363 colilike organisms from the soil (of Iowa) found the prevailing organisms to be of the 'nonfecal,' B. aerogenes, type. He has failed, however, to report a perfect correlation between the negative methyl-red test and positive V-P reaction as others have done. Of the 261 methyl-red negative organisms in his collection, only 84% gave a positive V-P reaction. Hulton[25] (1916) studied 45 coliform organisms of which only 12 were isolated from feces, and did not find any of the B. aerogenes type among the latter. The author concludes from this observation that the methyl-red negative, V-P positive reactions are of importance in determining colon organisms of fecal origin—a rather broad conclusion from an apparently limited study. A rather suggestive paper is one by Greenfield[21] on the soil and fecal types of the colon-aerogenes group in waters of Kansas. This author states in the account of her methods of isolation that all cultures that failed to liquefy gelatin were discarded. This is undoubtedly a misprint, and we assume it was meant that all gelatin liquefiers were discarded. This fact should be kept in mind for these organisms, representing the B. cloacae group, if included, would have increased the percentage of methyl-red negative, V-P positive organisms in the final results as reported.

The very interesting results obtained are as follows:

24% of 116 strains from raw waters were methyl-red negative
30% of 131 strains from treated waters were methyl-red negative
35% of 158 strains from ground waters were methyl-red negative

That is to say, the greater proportion of cultures studied were of the so-called true fecal type. On the other hand, 68% of 19 strains from natural ice were of the B. aerogenes group, which would seem to suggest that it may be more resistant to freezing temperature than the B. coli type.

In Table 1 are summarized the most significant findings in the work reported by various observers on feces and sewage, water, soil, grains, etc.

A brief survey of Table 1 indicates that the percentage of V-P +, M.R.— organisms in feces is not constant, and is, in general, small. The evidence also seems to show that on grains and cereals the B. aerogenes type is the predominating one. As to the prevalence of these types in soils, the facts are conflicting. Houston was unable to find any of the colon family in his studies in England. Clemesha was

[18] Levine, M.: Jour. Bacteriol., 1916c, 1, p. 153.
[19] Levine, M.: Jour. Infect. Dis., 1916a, 18, p. 358.
[20] Jour. Bacteriol., 1916, 1, p. 96.
[21] Jour. Infect. Dis., 1916, 19, p. 647.

able to isolate B. cloacae easily, while Burton and Rettger[22] found the predominating gas formers in soils to be apparently B. cloacae, not B. aerogenes. Except for the few researches recorded in the accompanying table, evidence in regard to the prevalence of these two types in waters of different sanitary quality is almost wholly lacking. Recently, the feeling has been growing that B. coli represents a true fecal type characteristic of undesirable pollution in waters, while the B. aerogenes type in water is derived from the soil, and represents a nonfecal source. It is perhaps fair to state that Rogers has carefully refrained from making such a broad statement, in spite of his very suggestive work, though Levine, Hulton and others apparently accept it as proven.

TABLE 1

SUMMARY OF RESULTS OF VARIOUS INVESTIGATORS IN REGARD TO THE PREVALENCE OF B. AEROGENES IN VARIOUS MATERIALS

Authors	Source	No. Strains	Per Cent. V-P+ or M. R.-
	Feces		
Ferreira, Horta, and Paredes (1908)	Human	117	6.8
MacConkey (1905)	Human	36	0
MacConkey (1909)	Human	178	6.2
MacConkey (1909)	Horse	67	11.9
	Other animals	87	0
Clemesha (1912)	Human	1,207	6.1
Clemesha (1912)	Cow	1,029	10.7
Rogers, Clark, and Evans (1914)	Cow	150	0.7
Rogers, Clark, and Lubs (1916)	Human	113	5.6
Rogers, Clark, and Lubs (unpublished)	Human	177	26.0
Levine (1916)	Sewage	...	23.0
	Feces	187	0
MacConkey (1909)	Cereals, waters, etc.	121	56.2
Houston, A. C. (1911)	Raw water	243	12.9
	Stored water	133	5.3
	Stored and filtered water	156	3.2
Rogers, Clark, and Evans (1915)	Grains	166	91.0
Rogers (1916)	Water	137	33.3
Greenfield (1916)	Water	405	30.0
Johnson (1916)	Soil	363	72.0*

* The percentage is figured on the results of the methyl-red test, not the V-P test.

This is the present state of the subject. The object of the present investigation was to add to the scanty evidence in regard to the relative prevalence of the B. coli and B. aerogenes types in waters of known sanitary quality in order to throw light on the significance to be attached to their presence. In other words, our object was to see if there exists a correlation between the character of the source and type of the coliform organisms isolated from waters in the neighborhood of New Haven, Conn.

[22] Jour. Infect. Dis., 1917, 21, p. 162.

Collection of Samples

The samples of water collected in this work were classified under three heads: (1) samples of water that were known to be polluted; (2) samples that were believed to be unpolluted, and (3) samples of stored raw waters used as municipal supplies. The decision as to whether a water is free from pollution or not is somewhat difficult, but our criteria were very strictly drawn to reduce the uncertainty to a minimum. The samples of unpolluted waters were taken from areas that were far removed from human habitation, and apparently from contamination from human or animal sources. One series for example was secured from Mt. Carmel, several hundred feet above the surrounding country, the samples being taken from rocky pools, or as the water flowed from under some overhanging sandy or rocky banks. In several cases, the drip was collected from precipitous earth banks covered with luxurious green growth. The possibility of human or animal contamination in such instances was very far removed. We might also record that these samples were taken during the autumn of 1916 when there was no very heavy rainfall, and therefore little chance of pollution being introduced by surface wash. The chance for contamination by air and dust is of course not excluded except in those samples taken directly from springs. Another set of samples was taken from a water-bearing area near Mt. Sandford, about 10 miles northwest of New Haven. This is situated several miles from any well-traveled roads, on a thickly wooded slope and well covered with underbrush. The probability of chance pollution here was highly remote. Most of these water samples were taken from springs as they bubbled out of the ground, thus offering no chance for even casual contamination by dust. This series constituted the greater proportion of the unpolluted samples. Others were taken throughout the country, from grassy woodland spring-pools, and at the headwaters of streams. In all cases a careful survey was made of the whole ground before a sample was accepted as fulfilling our requirements. To illustrate: at one place, we came across a piece of woods through which a crystal clear brook ran. It was far from any dwellings, etc. and seemed in many ways a desirable source. Several samples were taken at suitable points as we pursued our customary survey to the headwaters. We were, however, surprised to find a small pond dammed off near the origin of this stream, and on top of the steep banks, two dwellings and a stable, whose drainage provided part of the stream flow. We also learned that the pond was used as a swimming pool It is needless to say that our samples from this stream were discarded.

The samples of polluted water were easier to obtain. These comprised pools in barnyards, diluted sewage carried in the West and Quinnipiac Rivers, and ponds that very visibly received drainage from dwellings, barnyards, and privies along the banks. It should of course be kept in mind that the rivers carrying sewage also undoubtedly received drainage from unpolluted sources as well. We also included three cultures isolated from oysters taken in a polluted harbor. About two-thirds of the samples from stored lake and river waters were secured from the New Haven city water supply. Seven cultures were isolated during a routine examination of the city supply. The other samples were collected in the city at a short distance from the Maltby Lakes supply. This water was untreated, and came from a series of three large storage lakes of high sanitary quality. We encountered much difficulty in getting coliform organisms from this source in 100 c c samples and had to resort to concentration methods on larger volumes of water. This is rather surprising, as these samples were taken during the early spring when one would expect to find

sewage organisms most numerous in waters. The following apparatus was devised and proved quite effective as well as practical.

The apparatus used for concentrating the bacteria from this stored water was made up of galvanized iron fittings usually to be found in plumbers' shops. It consists of a 1¾ inch galvanized iron pipe of suitable length, threaded at both ends; a cap to fit the lower end and a reducer the upper. The cap has a ¼ inch hole bored in the center. The reducer tapers so as to fit a 1 inch threaded faucet (for connection of garden hose); and when not in use a 1 inch plug is used to close the tapered end. The upper end is closed with a perforated rubber stopper; and a porous clay filter of suitable size is inserted so that its outlet tube passes through the stopper and the hole in the cap when the cap is screwed on tightly. The outfit may thus be easily handled, autoclaved, and transported wherever necessary.

When it is desired to use the apparatus, the faucet from which the sample is to be secured is rendered sterile by flaming. The plug at the tapered end is removed aseptically, and the apparatus attached to the faucet. The water may now be turned on at full pressure for filtration. Because of its compactness and strength, the apparatus will effectively withstand a high pressure and in consequence give a high rate of filtration. We were able with a 6 inch Berkefeld candle to obtain a filtration rate of 1 liter in 5 minutes. A much higher rate no doubt might be obtained without danger. When the required volume of water has been filtered, the apparatus is unscrewed from the faucet, and the plug tightly replaced. The outfit may now be removed to the laboratory for examination. The water in the annular space (about 50 c c) is examined. The chamber is rinsed with sterile water, and the rinsings examined, and finally, the candle is removed aseptically and placed in a cylinder of lactose broth for enrichment and the resulting growth examined. As a rule we filtered 5 liters of water for a sample.

Fifteen cultures representing stored raw waters were obtained from the laboratory of the New Haven Water Company as isolated in routine examination. Eleven of the same type were obtained from the Connecticut State Board of Health Laboratory. These represent the only cultures that were not directly isolated by ourselves. They were isolated, however, by methods that were very closely, if not exactly, like our own.

Method of Isolation

The water to be examined was in all cases inoculated into various dilutions of standard lactose broth and incubated at 37 C. for 24-48 hours. The highest dilutions showing gas were streaked on azolitmin-lactose-agar plates which were then incubated at 37 C. These plates were examined from 12-24 hours later and acid colonies picked off and studied for further characteristics of the colon group. For reasons that have been clearly brought out by Prescott and Winslow (1913) the following characters were considered sufficient to permit an organism to enter our collection as a member of the colon-aerogenes group. The organism had to be a short, thick rod, non-spore-forming, aerobic and gram-negative. Further, it had to produce acid and gas in a lactose fermentation medium.

In our work we have assumed that the preliminary enrichment of the samples would not alter to any great extent the relative proportions of the two main types of coliform organisms should they both be present. MacConkey (1905) thinks that such a preliminary incubation in a liquid medium might alter the relations somewhat by favoring the growth of the B. lactis-aerogenes group. This point might be kept in mind when considering our own results on the

occurrence of the aerogenes types in these samples. The cultures were kept on standard nutrient agar slants at room temperature (about 18 C.) and transferred to fresh slants once a month. Tests made at the beginning and end of this research showed no apparent change in characters due to storage.

DISTRIBUTION OF COLIFORM ORGANISMS IN WATERS OF DIFFERENT TYPES

By these methods, we succeeded in isolating 255 strains distributed according to source as follows:

94 cultures from polluted waters
80 cultures from unpolluted waters
81 cultures from stored raw waters

It should be remembered that not all samples yielded cultures. This was especially the case with the samples from the unpolluted sources, about 100 samples proving negative. An important point is brought out by considering the dilutions from which these cultures were isolated in relation to the character of the source as given in Table 2.

TABLE 2

FREQUENCY OF ISOLATION FROM DIFFERENT DILUTIONS

	100 C C	10 C C	1 C C	1/10 C C	1/100 C C	1/1,000 C C	1/10,000 C C
Polluted, number.......	2	18	36	25	7	5	1
per cent.......	2	19	38	27	8	5	1
Unpolluted, number....	51	24	4	1			
per cent....	64	30	5	1			

The cultures from the stored waters are not included because of the different method of procedure in securing them. The greater number of cultures from unpolluted sources were isolated from 100 c c samples, and 94% of them came from 100 c c or 10 c c samples. On the other hand, in the polluted samples, most of the cultures were isolated from 1 c c and 0.1 c c, the incidence extending down to 0.0001 c c. These data testify both to the value of the test for the whole group of coliform organisms as a measure of pollution and to the validity of our judgment as to the actual sanitary quality of the waters examined.

CULTURAL AND BIOCHEMICAL CHARACTERS OF THE ORGANISMS STUDIED

Morphology and Motility.—Young 18-24 hour broth cultures were examined in hanging drop preparations. The majority of the cultures showed short thick rods with rounded ends. A few appeared rather slender. Some were exceedingly short and coccoid in shape. Motility was also observed in the hanging drop. About two-thirds of the collection were positively motile, and about equally distributed through the three classes of samples.

Gram Stain.—This was made on smears of the young cultures just mentioned. The stain was applied for 1 minute and the smear decolorized for 5 minutes. All the cultures in this series were gram-negative, although a few seemed to retain slight traces of the stain.

Indol Production.—The formation of indol in standard extract broth (Difco peptone) was determined after incubation at 37 C. for 4 days. The Ehrlich

test (para-dimethylamidobenzaldehyde + HCl) was used as it is said to be more reliable than the Salkowski test ($H_2SO_4 + KNO_2$). Sixty-seven, or 71% of the strains from polluted waters formed indol against 41, or 51% of those from unpolluted waters, and 57, or 62%, of those from stored waters. We do not know the exact significance of the indol test from the sanitary viewpoint. Houston places some reliance on it in determining his 'typical' coli organisms. Our results would indicate that the fewest indol producers are found in unpolluted waters, while the polluted sources give the most, the stored waters coming almost exactly in the middle between these two values.

Gelatin Liquefaction.—This was determined by spreading a loopful of 24-hour broth culture on the surface of nutrient gelatin and incubating at 20 C. for 30 days. Another series incubated for 10 days at 37 C. and then chilled in the ice-chest gave identical results. Of the strains from polluted waters 4, or 4%, liquefied gelatin, of those from unpolluted waters 2, or 2%, and of those from stored waters 4, or 5%. The gelatin liquefiers include the B. cloacae group, which is declared by Clemesha to be present in feces in varying numbers, but to be the most resistant type of coliform bacteria and capable of remaining for a long time in water and soils. This type occurred in very small numbers in our series and we are therefore unable to judge as to its probable significance.

Fermentation with Gas Production.—This was observed in standard lactose and glucose broths in Durham fermentation tubes incubated at 37 C. for 48 hours. All the cultures attacked these sugars with the formation of gas. Three of them, however, did so rather slowly.

Limiting Hydrogen Ion Concentration.—This was determined in the 0.5% Witte peptone, dipotassium phosphate-glucose medium of Clark and Lubs. (Incubation being at 37 C. for 4 days.) The final P_H was determined for each culture by the colorimetric procedure described by Clark and Lubs[3] (1917a). The standard phthalate-NaOH solutions were compared with Walpole's acetic acid-sodium acetate mixtures and checked up perfectly. The indicators used were methyl-red and brom-thymol blue. The methyl-red positive cultures gave a final P_H ranging from 4.6-5.4: the methyl-red negative cultures gave a final P_H from 6.4-7.2 in the methyl-red medium previously described. We encountered serious difficulties, however, when Difco peptone was inadvertently submitted for Witte's. A number of the cultures gave neutral tints with the methyl-red, while others of the B. aerogenes group produced a high limiting acidity. We have reason to believe that the degree of purity of the sugar may be another contributory factor in such peculiar behavior. Through the courtesy of Dr. Clark, who kindly furnished the crystalline materials, we were also enabled to run our cultures through the new synthetic medium described by Clark and Lubs.[23] The results were exactly the same as those obtained in the Witte's peptone medium, the only variation being that the ranges of the 2 types (methyl-red + and —) were placed closer together in the P_H scale. Thus, the methyl-red positives ran from 4.8-5.6 while the methyl-red negatives ran from 5.8-6.6. Several of the cultures gave neutral tints that were not easy to read, but the differentiation between the 2 types was quite clear. Two or three of the cultures did not grow as readily in this medium as in the other. In the main, however, we have found it to be exceedingly satisfactory for this work. In addition to the simplicity of preparation, there was an entire absence of inherent color after sterilization which is a desirable thing in colorimetric work. Classified according to the results obtained with the methyl-

[23] Bact. Abstr., 1917b, 1, p. 29.

red test, 72, or 77%, of the strains from polluted waters, 61, or 76%, of those from unpolluted waters, and 69, or 85%, of those from stored waters were positive, or of the B. coli type.

These results do not seem to indicate any close correlation between the source and type of coliform organism found therein. There seems to be an almost equal distribution of both types in polluted and unpolluted waters, and a somewhat greater proportion of the methyl-red positives in the stored waters. This is in fair agreement with the results obtained by Greenfield on the waters in Kansas. Our results also correspond in a way with the data we have quoted from Houston.[11]

The Voges-Proskauer Reaction.—This test was carried out in glucose broth which was incubated for 4 days at 37 C. by the addition of 10% NaOH, observing the development of the characteristic eosin-like coloration that gradually appeared. The tubes were not discarded as negative until a period of 24 hours had elapsed after addition of the reagent without the appearance of the coloration. Some of the tests were brilliant, but there were a number that were not so marked and left much to be desired. We must admit that if we had not been on the lookout for it in some of the B. aerogenes cultures, it would have gone by unnoticed. We have one culture (isolated from a polluted oyster) that persists in giving a positive V-P reaction, though it is a methyl-red positive organism. We have found, however, that there is in general an almost perfect correlation between the negative methyl-red test and a positive Voges-Proskauer reaction. The organisms giving a positive Voges-Proskauer made up 24% of the samples from polluted waters, 24% of those from unpolluted waters, and 15% of those from stored waters.

Rogers and his co-workers point out that there appears to be some difference between the typical B. aerogenes cultures isolated from grains and those from feces. They found that a large number of the grain cultures produced a noticeable pigment on nutrient agar and that only a small percentage of them attacked adonite. On the other hand, the B. aerogenes strains from feces produced only slight pigment and all vigorously attacked adonite. It therefore seemed desirable to test our B. aerogenes collection for these characters to see how far they agreed with Rogers' strains in relation to their source.

Pigment Formation.—The cultures were grown on agar for 15 days at 20 C. As much as possible of the growth was then removed with a spatula to white drawing paper and compared at once with the plates in Ridgway's "Color Standards and Nomenclature." With one exception, all the cultures produced no marked pigment. Most of them matched the "Cartridge Buff" or "Ivory Yellow" in the above plates. The exception was a culture isolated from a polluted brook running through a farmyard. It was also a strong gelatin liquefier. This culture gave a pigment one shade darker than "Honey Yellow" or 19″YO-Yi on Plate XXX.

Adonit Fermentation.—This was determined in a medium made according to the following formula: Water, 100 gm.; peptone (Difco), 1 gm.; beef extract, 0.4 gm.; dibasic potassium phosphate, 0.5 gm.; adonit, 1 gm. The cultures were incubated at 37 C for 6 days and then titrated. They were all methyl-red negative on addition of this indicator. We did not find any acid production where gas production was absent in this series. Of the B. aerogenes strains from polluted waters 13, or 59%, fermented adonit, of those from unpolluted waters, 17, or 90%, and of those from stored waters, 7, or 58%.

(The methyl-red positives were not tested for adonit fermentation.)

A greater proportion of the B. aerogenes from the unpolluted sources attacked adonit than did those from the polluted waters, although the numbers are too small to be significant. If the adonit fermentation may be taken as an index of B. aerogenes of fecal type, our results as far as they go would seem to show that the fecal type is prevalent in unpolluted sources. This is also borne out by the statement of Rogers[24] that there is a very much closer agreement in group characteristics between the B. aerogenes cultures from water and from feces than between the water and grain cultures.

CORRELATION OF BIO-CHEMICAL CHARACTERS IN THE SERIES OF CULTURES STUDIED

In the course of this study we have examined 255 strains of coliform organisms; of which 202 were methyl-red positive and 53 methyl-red negative. The correlation of other biochemical characters with the methyl-red reaction is indicated in Table 3.

TABLE 3

CORRELATION OF OTHER BIO-CHEMICAL CHARACTERS WITH THE METHYL-RED REACTION

Methyl-Red Reaction	Percentage of Cultures in Each Class			
	Voges-Proskauer Positive	Indol Formed	Adonit Fermented	Gelatin Liquefied
+	0.5	71.8		1.5
—	100.0	37.7	70.0	13.2

The Voges-Proskauer reaction is almost perfectly correlated with a negative methyl-red reaction, while indol formation is more common with the B. coli strains and gelatin liquefaction with the B. aerogenes strains, facts in close accord with the results of earlier workers.[25]

DISTRIBUTION OF B. COLI AND B. AEROGENES TYPES IN WATER OF DIFFERENT SANITARY QUALITY

In general our study, as indicated by Table 4, did not reveal any marked difference between the characters of coliform organisms from different sources.

Our study of a limited number of cultures isolated from polluted, nonpolluted, and stored waters does not therefore seem to show any connection between the type of organism and corresponding source. The final proof or disproof of any such connection must come from extended study in as many different parts of the country as possible. Hence, this study must not in any way be considered as definitive. We can only say that, under the conditions of our work at New Haven, no correlation between source and type of organism was discovered.

[24] Bact. Abstr., 1917, 1, p. 56.

[25] Jour. Infect. Dis., 1916, 19, p. 606.

An objection might be raised against our method of taking one single organism as representative of a sample, which is valid where the polluted samples are concerned. The cultures isolated from the nonpolluted sources are most important, however, and since no coliform organisms were obtainable in lower dilutions than 100 c.c. in a majority of these samples, we can fairly presume that an organism isolated from that volume of water, and not found in smaller volumes, must on the average come fairly close to being a representative of the prevailing type. The fact that of these organisms from unpolluted sources three quarters were of the B. coli type is therefore of significance. It would be highly desirable, however, to know the actual ratio of B. coli to B. aerogenes types in a given sample. To obtain data of this sort would be a time-consuming process, and we have not attempted it in this study.

TABLE 4

PRECENTAGE OF CULTURES FROM WATERS OF DIFFERENT QUALITIES EXHIBITING VARIOUS CHARACTERISTICS

Type of Water	Methyl-red Negative	Voges-Proskauer Positive	Indol Not Formed	Adonit* Not Fermented	Gelatin Liquefied
Polluted	23	24	29	41	5
Unpolluted	24	24	49	10	3
Stored	15	15	38	42	5

* This test applied only to 53 methyl-red negative strains.

That the B. coli type is the predominant one in feces and the B. aerogenes the predominant one on grains there seems no reason to doubt from the results of Rogers and his co-workers. What our data indicate, however, is that the grain or soil forms do not as a matter of fact gain access to the natural waters about New Haven in such numbers as to increase the relative prevalence of this type as compared with the conditions which obtain in more polluted waters.

We may of course rest safely on the general conclusion that the quantitative test for gas forming aerobes of all sorts is an admirable guide to the sanitary quality of water. Our own data tend to confirm this conclusion if any confirmation were necessary. If similar results to ours are obtained in other regions it would seem that the further differentiation between the B. coli and B. aerogenes may not be of special importance in determining the sanitary quality of water. If, however, in a given sample a large proportion of the B. aerogenes types were actually found, the results might perhaps be interpreted as signifying that the gas-forming organisms present were presumably not of recent fecal origin.

THERMOLABILITY OF SO-CALLED SYPHILITIC ANTIBODY

E. H. Ruediger

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

It is well known that unheated serums frequently give a stronger positive result with the Wassermann test than they do after they have been heated to 56 C. for 30 minutes. In order to avoid loss of complement-binding power, and for other reasons, Tschernogubow,[1] Hecht,[2] Noguchi,[3] Emery,[4] Gurd,[5] and others advocated the use of unheated human serum in the Wassermann test. Although it was shown that with unheated serums more positive results were obtained than with heated serums, none of the early workers attempted to measure the loss of complement-binding power incurred by heating serums to 56 C. for 30 minutes.

In this paper I shall present the results of Wassermann tests with heated and with unheated human serums. In none of these tests were native complement and hemolytic ambocepter used. Native complement was either removed by absorption or was ignored. Each human serum was tested 4 times, unheated with 0.05 c c per test tube, heated with 0.05 c c per test tube, heated and glycerolated with 0.05 c c (0.1 c c serum-glycerol mixture) of serum per test tube and heated and glycerolated with 0 125 c c (0.25 c c serum-glycerol mixture) of serum per test tube. Antigen, complement, hemolytic ambocepter, and blood corpuscles were used as described before.[6]

Test 1

Serums 1-30, inclusive, were tested within 24 hours after having been obtained from the patients. Nos. 1-15, inclusive, were obtained from nonsyphilitic patients, and Nos. 16-30, inclusive, from syphilitics under treatment. Each serum was divided into 3 portions, A, B, and C. Portion A was heated to 56 C. for 30 minutes. From Portion B the complement was absorbed in the following manner: Of 50% suspension of washed human blood corpuscles 0.75 c c was mixed with 0.75 c c of diluted antihuman hemolytic amboceptor

Received for publication Feb. 20, 1918.

[1] Deutsch. med. Wchnschr., 1909, 35, p. 668.
[2] Wien. klin. Wchnschr., 1909, 22, p. 338.
[3] Jour. Exper. Med., 1909, 11, p. 392.
[4] Lancet, London, 1910, 179, p. 732.
[5] Jour. Infect. Dis., 1911, 8, p. 427.
[6] Jour. Infect. Dis., 1917, 21, p. 502.

TABLE 1

SERUM WITH COMPLEMENT ABSORBED COMPARED WITH HEATED SERUM, NONGLYCEROLATED AND GLYCEROLATED

Number	Portions: A Heated Non-Glycerolated; B Unheated Complement Absorbed; C Heated Glycerolated	Serum per Tube C C	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results
1	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.5	+	+	±	+	+	±	Negative, —.
2	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
3	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
4	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
5	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
6	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	tr	Negative, —.
7	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
8	A	0.05	1	+	+	tr	+	+	tr	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.125	1.5	+	+	0	+	+	0	Negative, —.
9	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
10	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
11	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	tr	Negative, —.
12	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	tr	Negative, —.

TABLE 1—*Continued*

SERUM WITH COMPLEMENT ABSORBED COMPARED WITH HEATED SERUM, NONGLYCEROLATED AND GLYCEROLATED

Number	Portions A Heated Nonglycerolated, B Unheated Complement Absorbed, C Heated Glycerolated	Serum per Tube C C	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results
13	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	tr	Negative, —.
14	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
15	A	0.05	1.	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
16	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	0	+	+	±	Moderately positive, ++.
17	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	0	+	+	±	Moderately positive, ++.
18	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	0	+	+	±	Moderately positive, ++.
	C	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.125	1.5	+	+	0	+	+	±	Strongly positive, +++.
19	A	0.05	1	+	tr	0	+	+	±	Strongly positive, +++.
	B	0.05	1.25	0	0	0	+	+	±	Strongly positive, +++++ +++++.
	C	0.05	1.25	+	0	0	+	+	±	Strongly positive, +++++.
	C	0.125	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++.
20	A	0.05	1	+	+	tr	+	+	±	Weakly positive, +.
	B	0.05	1.25	+	0	0	+	+	±	Strongly positive, ++++.
	C	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.125	1.5	tr	0	0	+	+	tr	Strongly positive, +++ +++.
21	A	0.05	1	+	+	0	+	+	±	Moderately positive, ++.
	B	0.05	1.25	0	0	0	+	+	tr	Strongly positive, ++++ ++++.
	C	0.05	1.25	+	±	0	+	+	±	Strongly positive, +++.
	C	0.125	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++.
22	A	0.05	1	+	±	0	+	+	±	Strongly positive, +++.
	B	0.05	1.25	0	0	0	+	+	±	Strongly positive, +++++ +++++.
	C	0.05	1.25	+	±	0	+	+	±	Strongly positive, +++.
	C	0.125	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++.
23	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	0	+	+	±	Moderately positive, ++.

TABLE 1—*Continued*

SERUM WITH COMPLEMENT ABSORBED COMPARED WITH HEATED SERUM, NONGLYCEROLATED AND GLYCEROLATED

Number	Portions A {Heated Non-Glycerolated B {Unheated Complement Absorbed C {Heated Glycerolated	Serum per Tube C C	Ambo-ceptor per Tube Unit	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1′	2′	3′	
24	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	0	+	+	±	Moderately positive, ++.
25	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	0	+	+	±	Moderately positive, ++.
	C	0.05	1.25	+	+	±?	+	+	±	Faintly positive, ±.
	C	0.125	1.5	+	±	0	+	+	±	Strongly positive, +++.
26	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	0	+	+	±	Moderately positive, ++.
	C	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.125	1.5	+	±	0	+	+	±	Strongly positive, +++.
27	A	0.05	1	+	+	±?	+	+	±	Faintly positive, ±.
	B	0.05	1.25	+	±	0	+	+	±	Strongly positive, +++.
	C	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.125	1.5	+	0	0	+	+	±	Strongly positive, +++++.
28	A	0.05	1	+	±	0	+	+	±	Strongly positive, +++.
	B	0.05	1.25	0	0	0	+	+	±	Strongly positive, +++++. +++++
	C	0.05	1.25	+	tr	0	+	+	±	Strongly positive, ++++.
	C	0.125	1.5	0	0	0	+	+	±	Strongly positive, +++++. +++++
29	A	0.05	1	+	+	tr	+	+	±	Weakly positive, +.
	B	0.05	1.25	+	±	0	+	+	±	Strongly positive, +++.
	C	0.05	1.25	+	+	tr	+	−	±	Weakly positive, +.
	C	0.125	1.5	+	tr	0	+	+	±	Strongly positive, ++++.
30	A	0.05	1	+	+	0	+	+	±	Moderately positive, ++. +++
	B	0.05	1.25	±	0	0	+	+	±	Strongly positive, +++.
	C	0.05	1.25	+	+	0	+	+	±	Moderately positive, ++. ++++
	C	0.125	1.5	tr	0	0	+	+	±	Strongly positive, ++++.

representing 3 hemolytic units. This was allowed to stand at room temperature for 30 minutes. After having stood for 30 minutes 0.3 c c of the human serum was added, the mixture was well shaken, after another 30 minutes it was well centrifuged, and the clear fluid was used in the tests. Portion C was heated to 56 C. for 30 minutes and was mixed with an equal volume of sterilized glycerol. Portion C was tested in quantities of 0.05 c c and in quantities of 0.125 c c per test tube.

Table 1 shows the results obtained with Serums 1-30, inclusive. Serums 1-15, inclusive, gave negative results with all of the 4 methods used. With Serums 16-30, inclusive, the results varied greatly. Heated, glycerolated serum used in quantities of 0.125 c c per test tube gave the strongest positive results; the unheated serum came second;

TABLE 2

WHOLE SERUM COMPARED WITH HEATED SERUM, NONGLYCEROLATED AND GLYCEROLATED

Number	Portions A {Heated Non-Glycerolated B {Unheated Whole Serum C {Heated Glycerolated	Serum per Tube C C	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results
31	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
32	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	tr	Negative, —.
33	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.05	1.25	+	+	tr	+	+	tr	Negative, —.
	C	0.125	1.5	+	+	0	+	+	0	Negative, —.
34	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
35	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
36	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	tr	Negative, —.
37	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
38	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
39	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	tr	Negative, —.
40	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	±	+	+	±	Negative, —.
41	A	0.05	1	+	+	tr	+	+	tr	Negative, —.
	B	0.05	1.25	+	tr	0	+	+	±	Strongly positive, ++++.
	C	0.05	1.25	+	+	±?	+	+	±	Faintly positive, ±.
	C	0.125	1.5	+	0	0	+	+	±	Strongly positive, +++++.
42	A	0.05	1	+	+	tr	+	+	±	Weakly positive, +.
	B	0.05	1.25	±	0	0	+	+	±	+++ Strongly positive, +++.
	C	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.125	1.5	tr	0	0	+	+	±	++++ Strongly positive, ++++.

TABLE 2—*Continued*

WHOLE SERUM COMPARED WITH HEATED SERUM, NONGLYCEROLATED AND GLYCEROLATED

Number	Portions A {Heated Non-Glycerolated B {Unheated Whole Serum C {Heated Glycerolated	Serum per Tube C C	Ambo-ceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results
43	A	0.05	1	+	+	tr	+	+	±	Weakly positive, +.
	B	0.05	1.25	+	tr	0	+	+	±	Strongly positive, ++++.
	C	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.125	1.5	tr	0	0	+	+	tr	+++ Strongly positive, +++.
44	A	0.05	1	+	+	tr	+	+	±	Weakly positive, +.
	B	0.05	1.25	+	+	0	+	+	±	Moderately positive, ++.
	C	0.05	1.25	+	+	tr	+	+	±	Weakly positive, +.
	C	0.125	1.5	+	±	0	+	+	±	Strongly positive, +++.
45	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	−	±	+	+	±	Negative, —.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	+	tr	+	+	±	Weakly positive, +.
46	A	0.05	1	+	±	0	+	+	±	Strongly positive, +++.
	B	0.05	1.25	+	±	0	+	+	±	Strongly positive, +++.
	C	0.05	1.25	+	tr	0	+	+	±	Strongly positive, ++++.
	C	0.125	1.5	tr	0	0	+	+	±	Strongly positive, ++++. ++++
47	A	0.05	1	+	+	±	+	+	±	Negative, —.
	B	0.05	1.25	+	+	±?	+	+	±	Faintly positive, ±.
	C	0.05	1.25	+	+	±	+	+	±	Negative, —.
	C	0.125	1.5	+	tr	0	+	+	tr	Strongly positive, +++.
48	A	0.05	1	+	±	0	+	+	±	Strongly positive, +++.
	B	0.05	1.25	+	tr	0	+	+	±	Strongly positive, ++++.
	C	0.05	1.25	+	tr	0	+	+	±	Strongly positive, ++++.
	C	0.125	1.5	0	0	0	+	+	±	Strongly positive, +++++. +++++
49	A	0.05	1	+	+	0	+	+	±	Moderately positive, ++.
	B	0.05	1.25	±	0	0	+	+	±	+++ Strongly positive, +++.
	C	0.05	1.25	+	±	0	+	+	±	Strongly positive, +++.
	C	0.125	1.5	0	0	0	+	+	±	+++++ Strongly positive, +++++.
50	A	0.05	1	0	0	0	+	+	±	+++++ Strongly positive, +++++.
	B	0.05	1.25	0	0	0	+	+	±	+++++ Strongly positive, +++++.
	C	0.05	1.25	0	0	0	+	+	±	+++++ Strongly positive, +++++.
	C	0.125	1.5	0	0	0	+	+	±	+++++ Strongly positive, +++++.

Explanation.—+ means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis less than 60%; 0, no hemolysis.

heated, glycerolated serum used in quantities of 0.05 c c per test tube came third, and the weakest reactions were obtained with heated, non-glycerolated serum. A strongly positive and a negative result was obtained with Serum 25.

TEST 2

Serums 31-50, inclusive, were tested within 24 hours after having been obtained from the patients. Each serum was divided into three portions, A, B, and C. Portion A was heated to 56 C. for 30 minutes, Portion B was used whole, and Portion C was heated to 56 C. for 30 minutes and was mixed with an equal volume of sterilized glycerol. Portions A and B were used in quantities of 0.05 c c per test tube, and Portion C was used in quantities of 0.05 c c and 0.125 c c per test tube. Serums 31-40, inclusive, were obtained from nonsyphilitic persons, and Serums 41-50, inclusive, from syphilitics under treatment.

The results obtained in Test 2 are shown in Table 2. Serums 31-40, inclusive, gave negative results with all of the 4 methods used. With Serums 41-50, inclusive, the results varied greatly. Heated, glycerolated serum used in quantities of 0.125 c c per test tube gave the strongest positive results; unheated serum came second; heated, glycerolated serum in quantities of 0.05 c c per test tube came third, and plain heated serum gave the weakest reactions. Of Serum 41, Portion A gave a negative result, Portion B gave a strongly positive result, Portion C in quantities of 0.05 c c per test tube gave a faintly positive result, and Portion C in quantities of 0.125 c c per test tube gave a stronger positive result than did Portion B in quantities of 0.05 c c per test tube.

CONCLUSIONS

Unheated serum frequently gives a much stronger positive result with the Wassermann test than does heated serum.

The use of unheated serum is not practicable because it must be tested while still fresh, and because the complement present would interfere if one wished to use large quantities of serum.

In quantities of 0.05 c c of unheated human serum per test tube the native complement was not perceptible in the tests.

Quantities of 0.125 c c of heated, glycerolated serum per test tube gave stronger positive results than did quantities of 0.05 c c of unheated serum.

Heated, glycerolated serum gave a little stronger positive results than did heated, nonglycerated serum.

LIFE PHASES IN A BACTERIAL CULTURE

R. E. Buchanan

From the Bacteriological Laboratories of Iowa State College, Ames, Ia.

Several important contributions to our knowledge of the numbers of bacteria present in a culture medium at certain stages in the development of the culture have been made recently, but with the exception of the work of Slator (1917), apparently there has been no effort to coordinate these results and develop a complete mathematical theory of such changes in numbers. The present paper is an attempt to analyze the results of these authors and to present certain phases which have apparently been neglected heretofore.

When bacteria, particularly cells from an old culture, are inoculated into a suitable culture medium, as broth, the bacteria will at first remain unchanged in numbers; then multiplication begins, the numbers increase at first slowly, then more rapidly until a certain minimum average generation time is reached; this after a time begins to increase, and there is a negative acceleration in growth, which finally ceases; the numbers remain constant for a time, then the bacteria begin to die off.

Lane-Claypon[1] recognized four periods or phases in the life of a bacterial culture as follows:

1. Initial period of slow growth or even no growth.
2. Period of regular growth.
3. Period during which numbers remain more or less stationary.
4. Period during which the numbers of living bacteria are decreasing.

It would seem, however, that the life phases are somewhat more complex than indicated by the preceding statement. A study of the results secured by various authors indicates that seven relatively distinct periods may be differentiated. These may be recognized easily by plotting the logarithms of the numbers of bacteria against time. Chart 1 is such a plot with the seven phases indicated. It will be noted that points designating the beginning and end of each phase are points where a curve changes to a straight line and vice versa.

Received for publication Feb. 22, 1918.

[1] Jour. Hyg., 1909, 9, p. 239.

These various growth phases may be designated as follows:

1. *Initial Stationary Phase.*—During this phase the number of bacteria remains constant, and the plot is a straight line parallel to the x axis indicated by 1 — a.

2. *Lag Phase, or Positive Growth Acceleration Phase.*—During this phase the average rate of increase in numbers per organism increases with the time, giving rise to the curve a — b. This increase in rate of growth per organism does not continue indefinitely but only to a certain point determined by the average minimal generation time per organism under the conditions of the test.

3. *Logarithmic Growth Phase.*—During this phase the rate of increase per organism remains constant, in other words, the minimal average generation time is maintained throughout the period. This gives rise to the straight line b — c.

4. *Phase of Negative Growth Acceleration.*—During this phase the rate of growth per organism decreases, that is, the average generation time is increased.

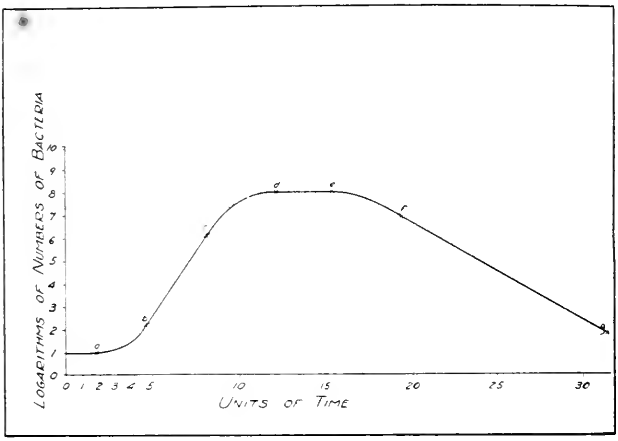


Chart 1.—Diagrammatic plot of logarithms of numbers of bacteria present in a culture.

The bacteria continue to increase in numbers, but less rapidly than during the logarithmic growth phase. This is the curve c — d.

5. *Maximum Stationary Phase.*—During this period there is practically no increase in the numbers of bacteria. The plot gives a straight line d — e parallel to the x axis. The rate of increase per organism is zero and the average generation time infinity.

6. *Phase of Accelerated Death.*—During this phase the numbers of bacteria are decreasing, slowly at first and with increasing rapidity, until the establishment of a logarithmic death rate. In the terminology used in the growth phases, the average "rate of death per organism" is increasing to a certain maximum. This gives the curve e — f.

7. *Logarithmic Death Phase.*—During this phase the "rate of death per organism" remains constant, the plot of the logarithms gives a straight line with a negative slope. This is represented by f — g.

If death and life in the bacterial cell could be regarded as reversible processes, we might expect the appearance of an eighth phase, a negative acceleration of the death rate.

It may be noted that the seven phases previously defined are in a sense arbitrary. The curve, if it could be plotted with data absolutely accurate, would probably be smooth; in other words, the portions designated as straight lines are probably curves, but with curvature so slight that they may be treated mathematically as straight lines without the introduction of any error commensurate in value with the inevitable experimental errors.

These various life phases of the bacterial culture will be discussed in some detail.

I. INITIAL STATIONARY PHASE

Spore-producing bacteria exhibit this growth phase particularly well. If a suspension of bacterial spores be placed in a suitable culture medium microscopic observation will show that growth does not apparently begin immediately. There can be no increase in numbers until the spores have germinated and begun to multiply. Samples of equal volume taken during this period show no increase in numbers. While this stage is most prominent with sporulating organisms it is by no means always absent in nonspore formers, as is shown by results of Lane-Claypon,[1] Penfold,[2] and others. In other words, there is evidence that in old cultures of many bacteria the cells are in a relatively dormant stage, the physiologic equivalent of sporulation though without the spore morphology. When such cells are planted in a suitable medium there will be an appreciable interval before a single cell will have resumed growth sufficiently to divide. During this phase the rate of increase per cell would be zero, and the average generation time infinity. The equation of the curve which represents this phase would be

$$b = B$$

where $b =$ Number of bacteria after time t,
$B =$ Initial number of bacteria.

Little work has been done on this phase. The conditions which determine its length are probably those influencing the length of other phases.

It should be noted that some cultures will not show this phase at all. If there are any actively dividing bacteria in the inoculum at the time of inoculation it will be absent or very transitory. It is therefore probable that the phase of the culture from which the transfer is made will affect the length of this phase.

[2] Jour. Hyg., 1914, 14, p. 215.

II. Lag Phase, or Phase of Positive Growth Acceleration

This phase apparently has not been differentiated from the preceding by previous writers. This is illustrated by the definition of latent period given by Chesney:[3] "By latent period or lag is meant the interval which elapses between the time of seeding and the time at which maximum rate of growth begins." The necessity for differentiation of the two phases is not urgent except when the first is long continued.

The lag phase may be defined as that period elapsing between the beginning of multiplication and the beginning of the maximum rate of increase per organism.

The phenomenon of bacterial lag was apparently first noted by Müller,[4] and was later studied by Rahn[5] and by Coplans.[6] Penfold[2] gave the first adequate discussion of the various theories which might be suggested to explain the phenomenon. Chesney[3] later made a careful study of the lag phase with special reference to the growth of the pneumococcus. A mathematical analysis of the lag phase was given by Penfold and Ledingham[7] and elaborated by Slator.[8]

Theories of Bacterial Lag.—Penfold[2] has enumerated some nine different theories as to the cause of bacterial lag, all of which he discards as inadequate. Inasmuch as certain of these have been maintained by other writers, and perhaps some discarded hastily, they will be briefly summarized and reasons for discarding given under the following seven heads.

1. The organism must excrete some essential substance into the medium before maximal growth can occur. Experiments show, however, that subcultures taken from cultures showing maximal growth do not show any lag period.

2. Adaptation to a new medium requires time. This must be discarded, inasmuch as transfers to the same medium may show lag.

3. Some of the bacteria transferred are not viable, and die off early. Inasmuch as enumeration is by plating and not by direct counting, the organisms not viable would never be enumerated.

4. Bacteria may agglutinate and plating would then be an enumeration of clumps and not of individual bacteria. While this may be a factor in some cases, it cannot explain the lag which still persists when adequate precautions against confusion from this source have been taken.

[3] Jour. Exper. Med., 1916, 24, p. 387.
[4] Ztschr. f. Hyg. u. Infektionskr., 1895, 20, p. 245.
[5] Centralbl. f. Bakteriol., 1906, Abt. 2, 16.
[6] Jour. of Path. and Bacteriol., 1909, 14, pp. 1-27.
[7] Jour. Hyg., 1914, 14, p. 242.
[8] Ibid., 1917, 16, p. 100.

5. Accumulated products of metabolism may injure the bacterial cell, the length of the lag phase is the time required to recover from the injury. Penfold rejects this explanation as inadequate. Chesney, however, insists that lag is "an expression of injury which the bacterial cell has sustained from its previous environment." This conception may well be an approximation of the truth, though probably not entirely accurate.

6. "The inoculum consists of organisms having individually different powers of growth, and during the lag the selection of the quick growing strain occurs in response to some selecting agent in the peptone." It is possible that this might occur in cultures which were not "pure lines," or which contained several strains, but there is no proof of its occurrence in pure strains. While there are undoubtedly some differences in the rates of multiplication of individual bacteria in the same culture, they are insufficient to account for the great differences characteristic of the lag period.

7. Bacteria must overcome an "inertia" before reaching maximal growth rate. Penfold dismisses this on the basis of certain experiments in which he chilled rapidly growing bacteria, and thus stopped multiplication, which was resumed at its former rate when the optimum temperature was restored. While it is probable that Penfold is correct in discarding inertia in this sense as a factor, nevertheless a modification of this theory is in the opinion of the writer the only adequate one suggested.

The explanation favored by Penfold is in fact a variant of the last. He believes that certain essential constituents of the bacterial protoplasm, probably synthesized in steps, must be present in the bacterial cell in optimum concentration or at least the intermediate bodies of the steps of the synthesis. When the bacteria cease growing these intermediate bodies diffuse from the cell and disappear, and before maximal growth can begin in a new medium these bodies must again be synthesized. This theory in effect holds that the loss of these substances gives rise to inertia. During the lag phase the bacteria are gradually recovering from injury.

It is probable that none of the preceding are wholly satisfactory explanations of the lag phase. An explanation more in accord with observed facts may be found in the assumption by the bacterial cells of a "rest period" comparable to the resting stages so often assumed by higher forms. It is a well known fact that at certain stages in the life history of many plants certain cells or tissues are developed which pass into a resting stage. When these are morphologically well differentiated they are termed spores, sclerotia, etc., in the lower forms, and seeds, bulbs, tubers, etc., among the higher types. In many other cases cells or tissues pass into a similar resting stage as a result of certain environmental influences, without showing marked morphologic differentiation. These resting cells are usually aroused to renewed growth and activity only as the result of certain stimuli. The cold of winter followed by the warmth of spring may be the stimulus which causes buds to develop. Some seeds will germinate only after the seed coat has decayed or has been scratched or corroded by acid. Bacterial spores form at certain stages in the life history of the bacteria, but do

not usually germinate in the parent culture in spite of abundant moisture, food and optimum temperature. Germination takes place under the stimulus of change to some new medium. It is altogether probable that most bacteria, whether spore producers or not, enter into such a resting stage. When not morphologically differentiated as a spore this resting period is probably more transitory than in a spore, but it is nevertheless just as real.

What happens, then, when a considerable number of bacteria in the resting stage are transferred to a medium suitable for development? If we were to examine the culture microscopically we would find that the bacteria would not all begin development at once, probably for the same reason that seeds placed under uniform favorable conditions for growth do not all germinate at the same instant. Cell division will occur in a few cells first, followed by larger and larger numbers at succeeding intervals of time until a maximum has been reached and passed, and at last all the cells have "germinated." As soon as a cell has actually germinated, there would seem to be no a priori reason why the cells should not thereafter multiply rapidly, showing practically at once a minimum generation time. There is no more reason to suppose that the length of time it takes a bacterial cell to germinate will affect its subsequent rate of growth than to assume that plants derived from seeds slow in sprouting grow more slowly than those from seeds soonest sprouted. After any cell had once "germinated" then, it would proceed to increase in numbers in geometrical progression. Theoretically the lag period would continue until the last viable cell had started to multiply; practically, however, it ceases before this as the rapid increase in the bacteria which have germinated soon makes the ungerminated cells such a small fraction of the whole number that their inclusion is within the limits of error of measurement of the numbers present.

MATHEMATICAL ANALYSIS OF THE LAG PERIOD

The lag period has been previously defined as that period during which there is an increase in the average rate of multiplication of the bacteria, an increase from zero to some constant which is maintained during the succeeding period. Another statement is that it includes the period during which there is a decrease in the average generation time. It should be noted that when used in this sense, the term generation time means the time required for the bacteria to double in numbers, if they continued growing at the same rate. At any given instant during this period there will be some cells not multiplying at all, these at that particular instant would have an infinite generation time, and the term average generation time would cease to have any meaning. In other words, during this period there is an acceleration in the rate of growth.

Let us first examine the equation of growth if the rate of increase per organism should remain constant, that is, the average generation time should not vary. Let

$$b = \text{number of bacteria after time } t,$$
$$B = \text{initial number of bacteria.}$$

It is evident that the rate of increase in number of bacteria at any instant will vary directly as the number of bacteria, or expressed in terms of the calculus:

$$db/dt = kb \text{ where } k \text{ is a constant. Now}$$
$$\frac{db/dt}{b} = k$$

Therefore k is the rate of growth per organism, or the velocity coefficient of growth. On integration this becomes,

$$\ln b = kt + \text{constant of integration.}$$

The constant of integration is found to be ln B by taking $t = 0$. The equation then becomes

$$\ln b/B = kt$$

This may be interpreted as the equation of a straight line, hence when lnb is plotted against t, a straight line with slope k will be secured.

The curve showing the number of bacteria after any time may be derived from the above equation

$$\ln b/B = kt$$
$$b/B = e^{kt}$$
$$\text{and} \quad b = Be^{kt}$$

This equation may be derived without resort to the calculus as follows:

$$\text{Let } n = \text{number of generations in time } t$$
$$g = \text{generation time.}$$

At the end of time t one organism will have produced 2^n bacteria, then

$$b = B2^n$$
$$\text{Now} \quad n = t/g$$
$$\therefore \quad b = B\,2^{t/g}$$
$$\text{Let} \quad 2^{1/g} = e^k$$
$$\text{then} \quad b = Be^{kt}$$
$$\text{and} \quad k = \ln 2/g$$

Since the rate of growth varies inversely as the generation time, k may be regarded as this rate of growth per organism, or the velocity coefficient.

Inasmuch as rate of growth per organism is a function of time, it is a matter of interest to determine just what the relationship may be existing between them. Penfold and Slator have suggested relationships empirically determined from experimental data. Apparently there has been no attempt to derive the relationship from theoretical considerations.

Assume that the lag phase represents the time required for all the viable bacteria planted to "germinate." Take as the time of "germination" the instant that the cell first divides to form two individuals. It is assumed that as soon as an organism begins dividing its rate of increase is at once constant. Let this be k′.

Let w = number of bacteria that are dividing after time t, = progeny of all bacteria that have germinated within time t

z = number of bacteria that have not germinated.

The rate of increase per organism $[f(t)]$ at any instant is given by the following equation:

$$f(t) = \frac{k'w}{w+z} = k'.\ 1/(1+z/w) \qquad (1)$$

It is apparent that if the numbers of bacteria "germinating" during each unit of time are plotted against time, a curve may be secured resting on the x axis at both ends, one of the forms of a probability curve. The general equation for such a curve has been shown by Karl Pearson to be

$$y = c(1 + x/a_1)^{m_1}(1 - x/a_2)^{m_2}$$

in which m_1 and m_2 are constants, c equals the maximum ordinate, and $-a_1$ and a_2 are intercepts of curve with x axis.

Let y be the number of bacteria germinating at time t, then

$$y = c(1 + t/a_1)^{m_1}(1 - t/a_2)^{m_2}$$

and $a_1 + a_2$ is the total length of the lag period.

The total number of bacteria which will germinate in time dt is ydt. Since the number of bacteria developing after time t from one organism is $2^{t/g}$, those which will develop after time t from those beginning growth during time dt is $y2^{t/g}dt$, and the total number of bacteria developed after time t from those starting growth within that time is

$$\int_{-a_1}^{t} y2^{t/g}dt$$

Therefore $w = \int_{-a_1}^{t} c(1 + t/a_1)^{m_1}(1 - t/a_2)^{m_2}\, 2^{t/g}dt$

The total number of bacteria which "germinate" within time t is

$$\int_{-a_1}^{t} ydt$$

The total number not germinated is

$$B - \int_{-a_1}^{t} ydt$$

$$\therefore\ z = B - \int_{-a_1}^{t} ydt$$

The relationship probably existing between rate of growth per organism and t may be shown by substituting the values secured for w and z in the equation (1).

$$f(t) = k' \frac{1}{1 + \dfrac{B - \int_{-a_1}^{t} c(1 + t/a_1)^{m_1}(1 - t/a_2)^{m_2}dt}{\int_{-a_1}^{t} c(1 + t/a_1)^{m_1}(1 - t/a_2)^{m_2}\, 2^{t/g}dt}}$$

All efforts to simplify this expression or put it into usable form have thus far failed. The only points where the exact relationship is known are when $t = 0$,

$f(t) = 0$, and when $t = a_1 + a_2$, $f(t) = k'$. It is evident that the relationship existing between rate of growth per organism and t during the lag period is quite complex.

The problem may also be attacked by the empirical derivation of a formula for a plotted curve by a critical examination of the data of the lag phase. This has been done by Ledingham and Penfold. These authors first reduced all figures to a seeding of 1, that is, the numbers of bacteria found at successive stages of the lag phase were divided by the initial number of bacteria. The logarithms of these numbers were plotted against the logarithms of times. This gave a curve which appeared to be logarithmic. The logarithms of the logarithms of the numbers of bacteria were then plotted against the logarithms of the times. These points were found to lie approximately on a straight line. If n is the slope of this line, and c the intercept with the x axis, the equation of the line is

$$n = \frac{\log (\log b)}{(\log t) - c} \qquad (2)$$

From this they derive the equation

$$t^n = k \log b$$

$$\text{Since} \quad \ln b = \ln 10. \log b$$
$$\ln b = \ln 10.t^n/k = k't^n$$

$$\text{where} \quad \frac{\ln 10}{k} = k'$$

Therefore $b = e^{k't^n}$ and for a seeding of B bacteria $b = Be^{k't^n}$
It is evident that this equation and the equation for regular growth

$$b = Be^{kt}$$

are special forms of the equation

$$b = Be^{\mu kt}$$

in which $\mu = f(t)$. In the equation for constant rate of growth per organism, $\mu = 1$, and in the Ledingham-Penfold equation $\mu = t^{n-1}$, and in the equation of initial stationary phase $\mu = 0$ and $b = B$. The equation developed,

$$b = Be^{k't^n}$$

has two constants which must be evaluated for each particular experiment. An equation of this general form was tested out by Ledingham and Penfold (1914) on data from eight series of experiments, and was found to give remarkably consistent results. The value of n in these experiments varied from 1.56 to 2.7 six being below 2.0. The value of k in the equation

$$t^n = k \log b/B,$$

varied from 2329 to 1,045,000.

The tables given by Chesney for increase of bacteria during the lag period afford an opportunity for testing independently the validity of the Ledingham-Penfold equation, or its generalized form.

Slator after a study of the data of Penfold (1914) concluded that in every experiment recorded there existed a relationship between the two constants n and k such that an equation could be derived in which there would appear

but one undetermined constant n. He found by examination that the following relationship always held:

$$\frac{\log k/n}{n} = \text{constant} = 2.024$$

$$k = 105.7^n$$

Substituting the value for k in the Ledingham-Penfold equation

$$t^n = k \log b \; B$$
$$t^n = n \; 105.7^n \log b/B = 105.7^n \log b^n/B^n$$

Slator uses the general form of equation

$$kt^n = \log b/B$$

This becomes $(.00945)^n t^n = \log b^n/B^n$.

While the equation as developed holds for the work of Penfold, Slator generalizes into the form

$$C^n t^n = \log b^n/B^n$$

in which C might have some value other than .00945. This can be put into the form of the equations

$$b^n = B^n 10^{C^n t^n}$$
$$\text{or} \quad b^n = B^n e^{k^n t^n}$$

The advantage of Slator's generalized equation over that of Ledingham and Penfold, at least for the lag period, is not apparent.

MEASUREMENT OF LAG

A numerical expression indicating the amount of lag may be secured in either of two principal ways: (*a*) An expression may be secured which will involve directly the length of the lag period, this may be termed "period of lag measurement;" (*b*) an expression may be secured which will give a numerical value to the degree of depression of rate of multiplication at any time during the progress of the lag period. This may be termed the "time index of lag."

(*a*) Period of Lag Measurement: Three suggestions have been made as to methods of measuring lag in terms involving the length of the lag period. These have been defined by Penfold.

1. The actual length of the lag period may be measured.

2. Coplans (1909) measured the restraint of growth in terms of minimum generation time. It may be expressed by the formula

$$\frac{t - ng}{g}$$

where t = length of lag period

n = number of generations during lag period.

g = minimum generation time.

3. The average generation time for the first part of the period may be compared with that of any succeeding period.

(*b*) Time Index of Lag: The degree or amount of lag at any instant during the lag period may have a numerical value assigned to it in either of two ways; the ratio of the generation time at any instant to the minimal generation time characteristic of the logarithmic period of increase may be determined, or, the rate of change or increase per organism at any given instant during the lag period may be compared with the similar rate of increase per organism during the logarithmic period. Inasmuch as the rate of growth must vary inversely as the generation time, it is evident that these two methods of expressing results will have a constant ratio.

1. *Measurement of Lag by Comparison of Generation Times.*—The problem is to secure the ratio of the generation time of the bacteria at any time during the lag phase to the minimal generation time. It should be recalled that the term generation time as used here is not a time average, but that length of time required for the bacteria present to double in number if the average rate increase per individual remained constant.

It was earlier developed that the expression

$$b = B\,2^{t/g}$$

represents the equation of growth if the rate of increase per individual remains constant. Differentiating and solving for g.

$$g = b \ln 2\ dt/db \qquad (1)$$

The value of dt/db may be determined for the lag phase by differentiation of either of the equations

$$b = Be^{kt^n} \qquad (2)$$

$$\text{or} \quad b^n = B^n e^{k^n t^n} \qquad (3)$$

Differentiating (2)

$$db/b = knt^{n-1}dt$$

$$dt/db = 1/bknt^{n-1} \qquad (4)$$

Substituting the value of dt/db in (1)

$$g = \frac{b \ln 2}{bknt^{n-1}} = \ln 2/knt^{n-1}$$

The ratio between the value of generation time as determined by this formula during the period of lag and the minimum value of g as determined during the logarithmic period gives a numerical index to the degree of lag at any instant.

If the equation

$$b^n = B^n e^{k^n t^n}$$

be chosen as the more general for the lag period (as developed from the work of Slator), the expression for generation time becomes:

$$g = \ln 2/k^n t^{n-1}$$

2. *Measurement of Lag by Comparisons of Rates of Increase Per Cell.*—The work of Slator suggests the possibility of measuring lag at any instant during the lag phase by a comparison of the rates of increase per cell with similar rates for the logarithmic period.

This may be determined from either lag phase equation

$$b = Be^{kt^n}$$

$$\text{or} \quad b^n = B^n e^{k^n t^n}$$

Differentiate

$$db/dt = bknt^{n-1}$$

The rate of increase per organism at any instant is therefore

$$\frac{db\ dt}{b} = knt^{n-1}$$

The corresponding rate of increase per organism during the logarithmic period is

$$\frac{db/dt}{b} = k'$$

The ratio knt^{n-1}/k' gives the numerical index desired.

If the second equation of the lag phase be employed the ratio becomes

$$k^n t^{n-1}/k'$$

It may be noted that the so-called "constant of growth" during the lag period, the expression $\frac{db/dt}{b}$, used by Slator and termed z is directly proportional to the μ of the equation

$$b = Be^{\mu kt}$$

III. THE LOGARITHMIC PHASE

The logarithmic phase of bacterial growth in a culture is that time during which there is a maximum rate of growth per organism, that is, the time during which a certain minimum generation time is maintained. The various relationships which define this period have for the most part been developed in the discussion of the lag phase. They are as follows:

If B = number of bacteria at beginning of logarithmic period,
b = number of bacteria after time t.
n = number of generations in time t.
g = generation time.
k = velocity coefficient of growth,

$$b = B\ 2^n = B\ 2^{t/g} = Be^{kt}$$

$$g = \frac{t \ln 2}{\ln b - \ln B}$$

$$n = \frac{\ln b - \ln B}{\ln 2}$$

$$k = 1/t.\ \ln.\ b/B$$

This phase of bacterial growth has perhaps been more investigated than any other. The mathematical relationships during this period are comparatively simple. It is evident that any effect of change of environmental conditions on the rate of increase of bacteria will be manifested through a change in the generation time. For every variable in the environment there is of course an optimum for each kind of organism, that is, a condition or concentration such that the generation time is minimal.

There is need for careful mathematical study of the effect of temperature changes, changes in concentration of nutrients, of hydrogen ions, of inhibiting substances, etc., on the rate of growth. It will be noted that the equation

$$k = 1/t \;.\; \ln \;.\; b/B$$

is one form of the expression for the value of the velocity coefficient of a monomolecular reaction. It has been shown that a similar (not identical) expression holds for the logarithmic death period of bacteria. Will the following expression

$$k = 1/tC^n \;.\; \ln b/B$$

hold where C is the concentration of some nutrient or inhibiting substance, and n a constant?

The temperature coefficient per degree or per 10 degree rise in temperature is in need of study, particularly near the minimum and maximum growth temperatures. This temperature coefficient over certain ranges has been determined for some bacteria. Lane-Claypon gives the value per 10° and 35° as 2 to 3 with B. coli. Similar results were secured from 20° to 30° by Hehewerth (1901) and Barber (1908).

IV. PHASE OF NEGATIVE ACCELERATION OF GROWTH

It is a matter of common laboratory observation that bacteria do not long maintain their maximum rate of growth, the logarithmic phase does not usually persist more than a few hours in quick growing types of bacteria. The average generation time apparently lengthens until at the close of the period the bacteria are no longer dividing.

The general equation of this portion can be written, as for the preceding phases

$$b = Be^{\mu k t}$$

During this phase the μ varies as some function of the time, from the 1 of the logarithmic period to 0. Apparently the exact relationship between μ and t during this phase has not been studied. It is apparent that as t increases μ must decrease, but a mathematical characterization has not been successful. The reasons for the decreased rate of growth per organism are complex. Among them may be enumerated the following:

1. The average rate of growth per cell will decrease with the increase in concentration of the injurious products of metabolism.

2. The average rate of growth per organism will decrease with decrease in the available food supply, or with some single limiting factor of this food supply.

3. As the period progresses a larger and larger proportion of the cells go into the "resting stage" and are withdrawn from those dividing or multiplying.

4. It is probable that before this period is completed some cells die.

Slator has suggested that the curve might be described by

$$b^n = B^n e^{ak^n t^n}$$

where n, k and a are constants suitably adjusted. Until further data are accumulated an attempt to evaluate these constants will prove difficult. From analogy with preceding and succeeding equations, it is possible that the growth equation of this phase might be

$$b = Be^{kt^{-n}} \text{ and } \mu = t^{-n-1}$$

V. THE MAXIMUM STATIONARY PHASE

During this period there is theoretically no change in the total number of bacteria present. If we still employ the useful general expression

$$b = Be^{\mu kt}$$

μ during this time remains zero, and the number of bacteria is constant.

Persistence of this phase must mean the balancing of increase and death. The rate of increase of bacteria must be such as to quite exactly make good the loss from death.

Investigations as to the length of this phase, and the influence of environment upon it are needed. With some organisms the phase is very transitory if it can be said truly to occur at all, with other forms apparently it persists for some time before there is marked any tendency to decrease in numbers.

VI. PHASE OF ACCELERATED DEATH RATE

Sooner or later the number of bacteria which die in a unit time will exceed the increase. In other words, as soon as bacteria reach the "resting stage" we may assume that they begin to die off, but they do not all reach this stage at the same instant. For some time there is an acceleration in the rate of death. The μk of the equation

$$b = Be^{\mu kt}$$

varies from zero to the velocity coefficient (constant) of the logarithmic death period. It also becomes negative in sign. It increases numerically in value during this period as time increases. During this stage the curve apparently is just the reverse of that of the lag period.

It is not improbable that the equation of the curve during this period will be found to be

$$b = Be^{-k't^n}$$

When $t = 0$, $b = B$. As t increases, b will be found to decrease more and more rapidly. Data are not at hand to prove the reliability of this equation. This stage probably does not persist long in most cultures, the velocity coefficient of death soon reaching a certain maximum.

VII. LOGARITHMIC DEATH PHASE

It was first shown by Madsen and Nyman and later by Chick that when bacteria are subjected to the action of unfavorable environment such as the presence of disinfectants they die off in accordance with the law which governs monomolecular reactions. If the logarithms of the numbers of surviving bacteria after various lengths of time are plotted against time, they will be found to lie on a straight line. The slope of this line is negative. This slope is the velocity coefficient of the reaction.

$$-k = 1/t \, . \, \ln b/B$$
$$\text{or} \quad k = 1/t \, . \, \ln B/b$$

The equation of the curve of the surviving bacteria is

$$b = Be^{-kt}$$
$$\text{or} \quad B = be^{kt}$$

This behavior of the bacteria has been abundantly verified by experimentation. It has been found to be of great service in the evaluation of disinfectants.

The effect of concentration of disinfectants has been developed principally by the work of Paul, Bierstein and Reuss, and of Chick[9] and the results generalized by Phelps. It is found that a change in the concentration of a particular disinfectant will change the velocity coefficient of the death rate in accordance with the following relationship:

$$k = k'C^n$$

where k' is the velocity coefficient of the original, and k the velocity coefficient with new concentration C, and n is a constant. For a different concentration the equation then becomes,

$$k' = \frac{1}{C^n t} \ln \frac{B}{b}$$

and the equation of the curve of surviving bacteria becomes

$$b = Be^{-kC^n t}$$

[9] Jour. Hyg., 1912, 12, p. 414.

Determination of the values of k and n for a disinfectant and a comparison of these values with those determined for some standard, as phenol, constitute efficient characterization of the disinfectant.

The Rideal-Walker and the Hygienic Laboratory phenol coefficients of disinfectants are determined by the use of facts inherent in these formulae. If the same concentration of two disinfectants are to be compared, we may place the same number, B of bacteria per unit of solution in each, and determine the length of time it takes to reduce the number of living bacteria to less than one per loop. Under these conditions the time required to change b to a certain number b′ is determined. The only undefined quantities left are t and k in the equations

$$b' = Be^{-k't'}$$
$$b' = Be^{-k''t''}$$
$$\therefore \quad k't' = k''t''$$
$$\text{or} \quad k'/k'' = t''/t'$$

that is, the velocity coefficients are inversely proportional to the times required for "disinfection." By determining variations in the values of these ratios with different concentrations one may approximate the values of n in the equation

$$b = Be^{-kC^n t}$$

If, in addition, the effect of heat be determined in accelerating the death rate of the bacteria, a relatively complete diagnosis of the characteristics of the disinfectant is at hand.

SUMMARY

1. There are at least seven life phases during the development of a culture of bacteria.

2. The general equation which represents the curve of the plot of numbers of bacteria against time is $b = Be^{\mu kt}$.

3. During the first or initial stationary stage μ is equal to zero, b is equal to B and there is no change in the numbers of bacteria.

4. During the second or lag phase μ is a function of time, increasing with time from 0 to 1. The relationship between μ and time is complex, but it is approximated by the equation

$$\mu = t^{n-1}$$

and the growth curve equation becomes

$$b = Be^{kt^n}$$

5. During the third or logarithmic growth phase $\mu = 1$ and the equation becomes

$$b = Be^{kt}$$

where k is related to the minimum generation time as follows:

$$k = \frac{\ln 2}{g}$$

and the equation of the growth curve is

$$b = Be^{kt} = Be^{(t \ln 2)/g}$$

6. During the fourth period or phase of negative growth acceleration μ decreases from the 1 of the logarithmic period of growth to 0. It is a function of time, decreasing with time. The relationship is complex, and no satisfactory evaluation of μ in terms of constants and time has been secured. It is possible the equation of growth may assume the form

$$b = Be^{kt^{-n}} \text{ and } \mu = t^{-n-1}$$

7. During the fifth period or maximum stationary phase μ remains equal to 0 and b equals B.

8. During the sixth period or phase of accelerated death rate μ varies from 0 to — 1. From analogy with the lag phase, the equation of growth during this phase may be

$$b = Be^{-kt^{n}}, \text{ and } \mu = -t^{n-1}$$

9. During the seventh period or phase of logarithmic decrease μ remains constant at -1, the growth curve having the equation

$$b = Be^{-kt}$$

10. The lag phase is interpreted as the time during which bacteria are gradually emerging from a resting stage. It is not improbable that the numbers of bacteria which emerge at various successive periods of time are distributed in accordance with some probability curve.

REPEATED AGGLUTINATION TESTS BY THE DREYER METHOD IN THE DIAGNOSIS OF ENTERIC FEVER IN INOCULATED PERSONS

EDWARD B. KRUMBHAAR, CAPT., M.R.C., U.S.A.

AND

PTE. W. B. SMITH, M.D., N.A., U.S.A.

From General Hospital, British Expeditionary Force, France

For the diagnosis of enteric fever (typhoid and paratyphoid A and B fevers), both agglutination tests and cultures from blood, urine or stool have long been considered indispensable. A positive culture from any of these sources is of course absolute proof of the existence of active infection (or of carrier in the case of the urine or stool), and should be tried in doubtful cases as many times as possible; but unfortunately, it has not proved possible under usual clinical conditions, to obtain positive results in a very high percentage of cases. In special investigations, to be sure, positive blood cultures have been obtained in two-thirds of all cases tested,[1] or in even more if taken early in the disease; positive urine cultures in about 25% of all patients[2] and positive stool cultures in from 10-81% of cases,[3] the higher figures being obtained late in the disease. It is widely admitted, however, that the routine results even of the best clinical laboratories fall far short of these favorable figures, and it must now further be recognized that under certain unfavorable conditions, even less satisfactory cultural results may be expected. One such condition is the great prevalence of antityphoid inoculation, which not only distorts the accepted clinical picture of this already "protean" disease, but which a priori should make positive cultures more difficult to obtain. The best evidence on this point (from unpublished statistics) indicates that positive blood cultures are only obtained with half the frequency in inoculated individuals (11 vs. 22%), but the figures for positive urine and feces cultures are about the same in both inoculated and uninoculated. In military service, in which the proper diagnosis of enteric fever is today of prime importance, the exigencies of active

[1] Coleman and Buxton: Am. Jour. Med. Sc., 1907, 133, p. 896.

[2] Hiss and Zinsser: Textbook of Bacteriology, 1916, p. 411.

[3] Hiss: Medical News, 1901, 78, p. 728.

service, with the necessarily frequent removal of patients and lack of adequate facilities near the front, beset the cultural diagnosis with still further difficulties. One or more cultures should always be taken from the blood, however, if the case is seen early; or from the urine or stool, if late in the course of the disease.

The importance of agglutination tests, therefore, would be at once obvious, were it not for the difficulties that antityphoid inoculation here also presents. Former methods of making a diagnosis after a single and but slightly quantitative test have now had to be abandoned as worthless, because, as is well known, specific agglutinins are present for many months in the blood of inoculated individuals. As the agglutinin titer after infection or inoculation follows a definite curve, however, it is possible to determine whether repeated tests in a given case follow the steady line resulting from a more or less remote inoculation or the sharp variation of a recent and still active infection. As Glynn[4] has recently pointed out, "the necessity of repeated serologic tests in soldiers suspected to have typhoid, but previously inoculated against it, was suggested in India in 1910 by officers of the R. A. M. C.,[5] Dreyer,[6] Walker[7] and others should also be credited with emphasizing the need of repeated tests, and simplifying and standardizing the technic. It has been shown that whereas agglutinin formation does not occur until the 2nd week of the disease, a sharp rise then occurs, which reaches its maximum between the 16th and 24th day. (Both Glynn and Dreyer have found typhoid agglutinins in dilutions as high as 1:200,000.) (It is well to note that in abortive cases this maximum may not be reached until after the temperature has returned to normal.) This is followed by a sudden drop usually lasting several days, which tapers off into a curve that gradually drops for several months to zero.

The diagnostic value of repeated tests by Dreyer's method, it is the purpose to examine in the present paper, pointing out some of its limitations and dangers, and including the results of some experiments on healthy inoculated individuals. As at present practiced in the British Army, the test is made by agglutinating the patient's serum against "Standard Agglutinable Cultures," prepared solely by the

[4] Glynn, and others: Report on 2,360 Enteritis Convalescents. Med. Res. Com. Spec. Series, 1907, 7, p. 48.

[5] Annual Reports of Divisional Sanitary Officers and Enteric Depots, 1910, Simla.

[6] Proc. Roy. Soc. Med., 1915-1916, 9, p. 11, Med. Sec., p. 1.

[7] Lancet, 1915, 1, p. 324.

Department of Pathology of Oxford University. In addition to the greater ease and economy of this centralization of culture preparation, it theoretically allows results obtained in one laboratory to be compared with later results on the same patient that may have been obtained in a different laboratory. As will be shown later, however, the many factors that enter into the final reading play such an important part that results from different laboratories should only be relied on to a certain — or rather to a somewhat uncertain — degree.

Notes on Technic

Practical experience with the method indicates certain phases in the procedure that should be dealt with in detail:

1. The person obtaining the blood should be sure of getting a liberal supply. More than twice as much as for the microscopic test is needed to secure the necessary 6 drops of serum. If insufficient blood is obtained, or Dreyer's pipet is not at hand, an alternate "volume" method with an ordinary pipet and rubber teat may be employed. In this method the available serum is drawn up to a grease or file mark on the pipet, and a 1 : 10 dilution obtained by mixing with 9 volumes of diluent drawn to the same mark. An appropriate amount of diluted serum is now drawn to a mark higher on the pipet, discharged into tube 1 of the series and mixed with an equal amount of water. One half of this dilution (i. e., to the higher mark) is then withdrawn, deposited in the next tube, mixed with an equal amount of water and so on through the series. The process is repeated with the second and third rows with appropriate cleansing of the pipet and finally an equal amount of the standard agglutinable culture (i. e., to the higher mark) added to each tube. This gives a final dilution of 1 : 40, 1 : 80, etc., but the dilution may of course be varied to suit individual cases. The grease marks should be high enough on the pipet to ensure that the final dilution at least half fills the tube.

2. The agglutination cultures should always be practically transparent (i. e., not contaminated), and should never be acid. It is stated by the Oxford Standard Laboratory that "the growth of a mould in a bottle does not affect the agglutinability of the culture. If the mould be fished out and a drop or 2 of chloroform be added to the fluid to prevent further growth, the culture is as good as ever. Bacterial growths in the cultures also occur, but are rare and almost invariably the result of careless handling." In our experience, however, contaminations of both kinds are common in spite of careful handling, unless a given set of bottles is used up in a few days. To lessen the chance of contamination, the approximately requisite amount is poured into watch glasses for use and the excess discarded. It has been found expedient also to discard all cultures showing signs of contamination. We have found by comparative tests that unaccountably low agglutination readings can usually be traced to spoiled cultures.

3. Although the directions call for dilutions of serum with normal salt solution, it has been the custom with many pathologists using this method to use distilled water instead. It is thought that sharper results are obtained, and we understand that either diluent is permitted by the British authorities. We have

[5] Jour. Path. and Bact., 1909, 13, p. 331.

made some comparisons of readings obtained with salt solution and water, and find that readings of the latter average almost a whole tube higher. Thus in 39 tests of both methods, a total of 28 higher tubes was obtained with distilled water as diluent, when read with the naked eye; of 32 when a hand glass was used. In only 12 were similar readings obtained and in 2 the salt solution gave 1 tube higher reading. We, therefore, feel that only one diluent should be used by every one, and as the flocculi seem more distinct with distilled water, would prefer the use of the latter.

4. Another variation in technic which is not only considered permissible, but is practiced by many users of the method, is the use of a hand glass in making the readings. We have compared readings in over 400 tubes, using a hand glass of 2 diameters, and have found that in the majority of cases, higher readings of from 1-5 tubes are obtained. The difference is less marked with B. typhosus (28 tubes in 43 comparisons), than it is with the paratyphoid (60 tubes in 39 comparisons of A and 61 tubes in 42 comparisons of B). This is probably due to the normally greater size of the typhosus clumps when the Oxford Standard Agglutinable Cultures are used. The reading with the hand glass seems preferable, as it allows detection of true agglutination to its furthest limits. The flocculi have the characteristic appearance, still higher readings are not obtained if a more powerful glass is used, and if the test is allowed to stand over night, the "naked eye" readings approximate the previous "hand glass" readings closer than they do those of the previous "naked eye" readings.

5. If there is any doubt about an individual reading (particularly if in the B group), or if circumstances will permit, it is advisable to let the Dreyer stand wait over night and make a second reading the next morning. If these subsequent readings are higher they should be included in the final report.

6. All tubes of the series should be examined, because it sometimes happens (especially in the paratyphoid series), that the stronger dilutions of serum will not agglutinate as well as the weaker ones. The over-night reading, especially the para B tests, will also obviate mistakes due to this cause.

7. It has recently been suggested that the best results are obtained if the height of the water in the bath is such that one-half of the mixture in the tubes is in and one-half out of water. We have made no tests of the value of this detail. It is convenient to have a water bath of distilled water and in a covered container, so that dirt or calcium deposits on the tubes will not interfere with the reading.

8. If the regulation Dreyer pipet is used it is important that the same pipet or another of the same external caliber should be used throughout the test. Donald[9] has shown that the size of the drop varies directly with the external diameter of the tip. Inasmuch as the drop becomes smaller the more horizontal the pipet is held, the angle at which the pipet is held should be the same throughout the operation. There is no appreciable difference in the size when the pipet is held at 80 or 90° (Donald[10] and Garrow[11]).

9. Other simplications in the use of the Dreyer pipet are included in the following letter from Professor Glynn: "The 6 drops of serum to be tested are distributed from a clean and absolutely dry pipet. The surplus serum is then discharged and the pipet washed out 3 or 4 times with diluent, namely,

[9] Lancet, 1816, 2, p, 423.
[10] Ibid., p. 994.
[11] Ibid., p. 864.

water, 54 drops of which are added to make the dilution of 1:10; there is no use to wash and dry the pipet first. The wet pipet is now used for distributing the water in the small dilution tubes. The surplus water is then blown out of the teat, and by shaking the pipet with the hand any drop on its end is removed. The wet pipet, which now contains inside and out about one-third of a drop of water — this I have ascertained by weighing — is rinsed out in the mixture of serum and water, which is then distributed with it into the small dilution tubes; the addition of a third of a drop of water to 60 is of no practical importance. The pipet is again washed out with water 3 or 4 times, emptied, and shaken as before, and then rinsed out in the bacillary emulsion, which is next distributed in the dilution tubes. In order to save the time of counting the drops of bacillary emulsion it is a very simple matter to calibrate the pipet to hold the right amount by marking the glass with a file. If care is taken to select pipets which are all approximately the same size as measured by a gauge, then one calibrated pipet can always be used for typhoid emulsion only, another for the paratyphoid A, and a third for paratyphoid B. This saves still more time. In fact, instead of washing out the pipet with 3 different fluids and drying on 6 occasions (for a single test against 1 bacillus) it is then only necessary to wash the pipet 3 or 4 times with 1 fluid on 1 occasion to make a single test with 3 different emulsions, excluding the final washing and drying."

TABLE 1

COMPARATIVE AGGLUTINATIONS IN DIFFERENT LABORATORIES

Hospital Number	Patient 1			Patient 2		
	Typhoid	Paratyphoid A	Paratyphoid B	Typhoid	Paratyphoid A	Paratyphoid B
1	1:250	1:500	1:125	1:1000	1:1000	1:250
2*	1:500	1:250	1:125	1:1000	1:1000	1:500
3	1:250	1:50	1:25	1:125	1:250	0
4	1:640	1:160	0	1:80	1:40	0

* A sample of the blood of Patient 2 done the next day at this hospital with different cultures gave T. 1:250; A. 1:250; B. 1:125. Reduced to agglutinin units the differenees are slightly greater.

10. The best and most consistent results are naturally obtained when the tests are done by the same observer using the same batch of cultures. If successive tests on a given patient are done in different laboratories, allowance must be made for a greater factor of variation in the method. This is not only due to the "personal equation" that enters into most laboratory tests, and to possible slight variations in the various batches of the standard cultures, but also to the remediable lack of uniformity in the various details just discussed. If, for instance, one observer should happen to use salt solution as a diluent, and make an immediate reading with the naked eye he might easily get a reading 3 tubes lower than another observer using distilled water and reading the next morning with a hand glass (e. g., a difference of 1:25 and 1:250). That this is not an imaginary situation is shown by the following control tests done simultaneously on the same blood by 4 pathologists in adjacent laboratories.

As is indicated by the first sentence in the footnote, it is very probable that variations in the different batches of agglutinable cultures were responsible for at least some of these discrepancies. Such figures do not indicate that comparisons from different laboratories are valueless, especially as in active infection the

rise in agglutinins amounts to even greater differences than are here involved; but they do mean that all steps must be carefully standardized and that even then too much reliance must not be placed on small fluctuations. Differences of 1 tube (e. g., between 1 : 50 and 1 : 125) should hardly be taken into consideration, and in some cases even greater fluctuations do not form sufficient basis for positive diagnosis.

11. The directions for placing "Standard Agglutination" midway between 2 dilutions under certain conditions have been still further amplified in the following interpolation table and factors, depending on the character of the sediment or flocculi as seen by the naked eye.

TABLE 2

DREYER'S INTERPOLATION TABLES AND FACTORS

Agglutination	Factors
Total (i. e. all sedimented)	1.47
Total (mostly sedimented, few flocculi)	1.29
Standard (very large flocculi, slight if any sediment)	1.19
Standard (large flocculi, no sediment)	1.13
Standard	1.00
Standard (flocculi smaller)	0.88
Trace (large numbers of small flocculi)	0.77
Trace (many very minute flocculi)	0.68
Trace (if very slightest granules appear)	0.60
Trace ("a very doubtful tube indeed")	0.53

We have found, however, that the maximum positive readings (with a hand glass, at any rate) are always in the "trace" group. The differences involved are, therefore, so much less than those introduced by other variable factors that the use of these tables only seems to detract from the simplicity of the test without adding to its accuracy. Also a comparison of results obtained with different batches of cultures has given us the impression that the character of the flocculi may vary with different batches, so that for this reason also the soundest policy is to rely only on the maximum dilution in which agglutination is detectable.

INTERPRETATION OF RESULTS

In interpretation of results, a few points in addition to those given under the head of "diagnosis" in the directions are useful. When 3 or more tests have been made and the results translated into agglutinin units, the curve obtained must be interpreted both according to the date of onset and to the date, number and kind of inoculations received. The date of onset should be elicited by careful questioning. If the maximum agglutinin titer apparently falls outside the 16-24 day limit, the date of onset should be again investigated. If it really falls outside, a positive diagnosis should be avoided, even with a rise of 100-200%, as other fevers may cause similar fluctuations. It should also be remembered that 2 successive equal observations may have missed an intervening maximum and that an apparent maximum is rarely the true highest point reached. In allowing for the effect of inoculation,

though each individual's agglutinins respond to inoculation with different intensity, nevertheless, the general direction of the curve is the same in all, so that a low response shortly after inoculation, or a high one many months after, point in themselves to absence or presence of infection, respectively. Of course, if only typhoid vaccine has been given, even low agglutination in either paratyphoid series has diagnostic importance. There is some evidence that other fevers may reduce inoculation agglutinins, but this point still remains undecided and has not been investigated by us. Finally, if, as is the case in the British Expeditionary Forces, the ultimate diagnosis rests with the pathologist, he should give careful consideration in consultation with the clinician to the clinical aspect of the case. It has been found advisable and very useful to make use of the term "Enteric Group" for those cases whose dates are very suggestive, but not sufficiently concise to warrant the more accurate diagnosis of any one of the three.

AGGLUTININ CURVES IN T. A. B. INOCULATED INDIVIDUALS

Advantage was taken of recent T. A. B. inoculations in previously inoculated individuals to study the resulting agglutination curves. In the following charts are given the average curve (composite agglutinogram) obtained from 40 such individuals, also an example of one of the maximum and one of the minimum responses. As the former had been proved not to be a carrier by repeated negative stool examinations, and as neither were isolated instances, they may be taken as showing the marked variations of individual response to inoculation that may be expected. The series were also tested one month after inoculation, but as the results averaged lower than in the following month (i. e., obviously incorrect) they were not included. They are mentioned here to show how incorrect may be the results obtained by those not sufficiently familiar with the methods and its pitfalls. The high but quick falling B. curve, the lower and steadier A. curve, and the intermediate T. curve are similar to results obtained by other observers (Garrow[8]). Lack of time prevented further elaboration of the curves, but they would doubtless have dropped slowly for many months. Garrow, for instance, found an average T. agglutination of 1:40 seven months after inoculation; and of 1:20 sixteen months after.

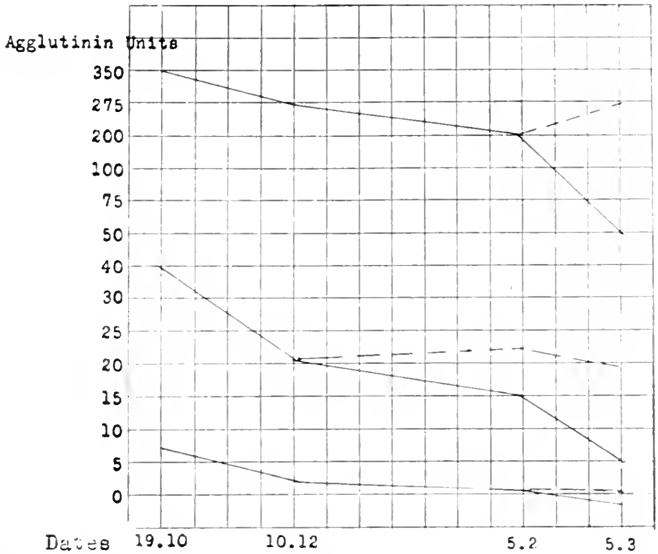


Chart 1.—B. typhosus agglutinograms after T. A. B. inoculation. In this and the following 2 charts, an average curve of the agglutinin units of 40 individuals is given, also sample curves of high and low responses. Three doses of U. S. Government vaccine were given at 10-day intervals 2 months before the first test recorded. The usual dose of 0.5, 1, and 1 c c were given, each c c containing 1,000 million typhoid and 750 million of each paratyphoid bacilli. In all charts dotted lines indicate "hand glass reading."

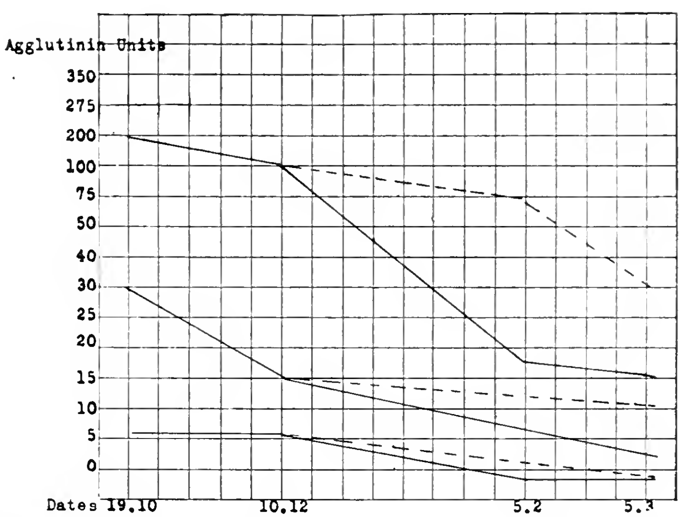


Chart 2.—B. paratyphosus A agglutinograms after T. A. B. inoculation. Same details as Chart 1.

EFFECT OF PARA B INOCULATION IN T. A. INOCULATED INDIVIDUALS

As some of the personnel of this unit had received only typhoid and paratyphoid A inoculation in the spring of 1917, an opportunity was offered when they received para B inoculation in January, 1918, to study the effect of the latter on the T. and A. agglutinins. The accompanying charts show that in all but one case a very distinct but evanescent rise of typhoid agglutinins was produced, whereas the changes in the para A agglutinins were so insignificant that they are

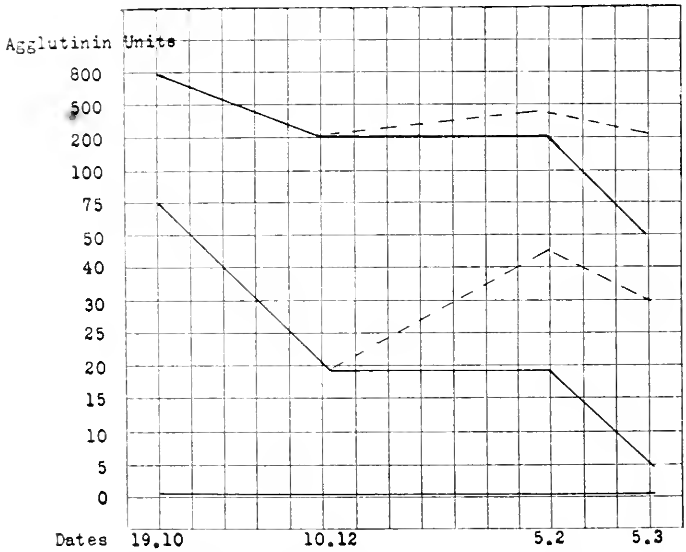


Chart 3.—B. paratyphosus B agglutinograms after T. A. B. inoculation. Same details as Chart 1.

well within the limits of error of the method. This corresponds with other experiences that the agglutination reaction with para A tends to be erratic. From these and similar observations of other observers, it is proper to deduce that paratyphoid fever, like other less related infections, will probably cause a temporary rise in the inoculation typhoid agglutinins (partial, minor or meta agglutinins), and proper allowance must be made for this in the interpretation of agglutinin curves of suspected enteric fevers. Dreyer, for instance, has found that "if an animal inoculated some weeks before with a micro-

organism, be inoculated with a nonlethal dose of vaccine of another kind, its agglutination titer for the first rises and preserves the same course as the original again." Garrow also showed that 60 of 98 typhoid inoculated individuals showed an increase of from 300-6,300%, 10 days after para A and B inoculation. It is interesting also to note that the highest rise in typhoid agglutinins occurred in the test made 3 days after the second inoculation, and 13 days after the first; in other words, that the first inoculation exerted the most stimulating influence and that thereafter (as it was then too early for the second inoculation to have had effect) the agglutinin titer dropped in spite of the next 2 inoculations.

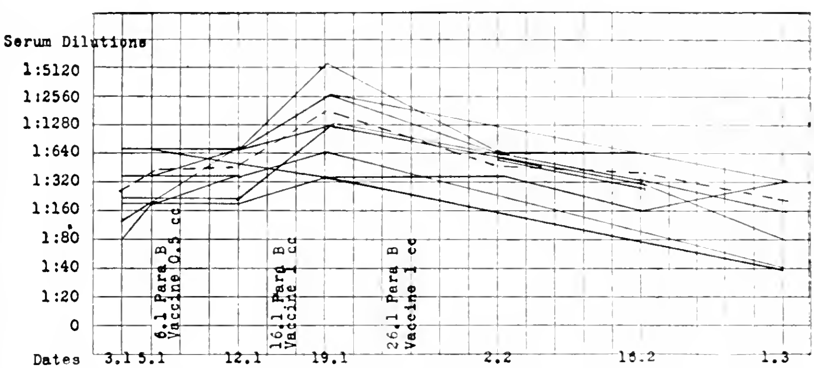


Chart 4.—B. typhosus agglutinograms in T. A. inoculated individuals after inoculation with paratyphoid B vaccine. In this and the following 2 charts individual curves of 8 persons and an average curve are given. They all had received T. and A. vaccine separately 8 months before (i. e., a total of 5,000 million in 5 doses) and received 3 doses of B. vaccine (i. e., 2,500 million in 3 doses) at the time indicated on the chart. Note the nonspecific rise in T. agglutinins. As the agglutinin factors were the same throughout these series, the maximum positive dilutions are given instead of agglutinin units, as in the former charts.

The agglutination tests with para B cultures show that whereas no agglutination was detected before inoculation, B agglutinins had begun to appear in half the cases by the sixth day, and the rest by the twelfth day after inoculation. Here again a maximum titer was quickly reached and the curve began to fall in some cases before the third inoculation had been given. This is in the main similar to the experiences of Garrow[12] who considers 5 phases of agglutination after inoculation: (1) A latent period of 4 or 5 days; (2) a rising period of 7 days; (3) a maximum reached on the 12th day; (4) a

[12] Jour. Roy. Army Med. Corps, 1917, 29, p. 412.

rapidly falling period lasting to about the 24th day; (5) a residual period lasting many months.

Only once did the third inoculation have any influence in determining the maximum titer. This differs from Craig's[13] experiences, where after T. A. B. inoculation the highest titer was always reached shortly after the 3rd weekly inoculation. The behavior of the agglutinin

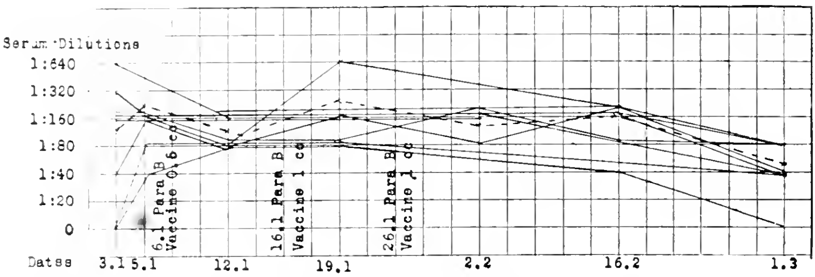


Chart 5.—B. paratyphoid A agglutinograms in T. A. inoculated individuals after inoculation with paratyphoid B vaccine. Note that no appreciable change is caused.

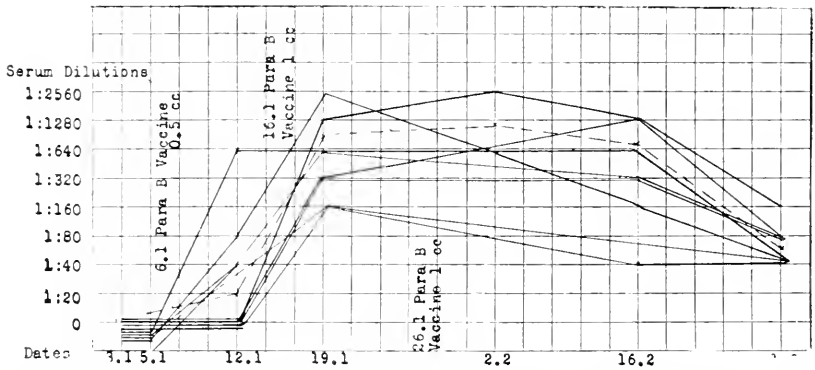


Chart 6.—B. paratyphosus B agglutinograms in T. A. inoculated individuals after inoculation with paratyphoid B vaccine. Note the incubation period and the sharp but partially evanescent rise in agglutinins.

curve during the second and third inoculations might be interpreted either as a depression resulting from the repetition of the dosages, or as such an extreme response to the dose that fell on a virgin field that the responses to the later doses were masked by the decline from the earlier dose. A single opportunity was offered to test this by repeating

[13] Jour. Am. Med. Assn., 1917, 59, p. 1000.

the inoculations at longer intervals. As the curve in this case showed a similar fall to the majority of the earlier cases, it seems as if the latter assumption was correct.

CONCLUSIONS

The diagnosis of fevers of the enteric group may be made by agglutination tests in many cases when cultures have been negative, but the test should never be used as a substitute for cultures.

In inoculated individuals the diagnosis may be made by quantitative agglutination tests, if 3 or more tests are made at suitable intervals and the resulting curve interpreted in the light of the inoculation and clinical data and the date of onset of the disease.

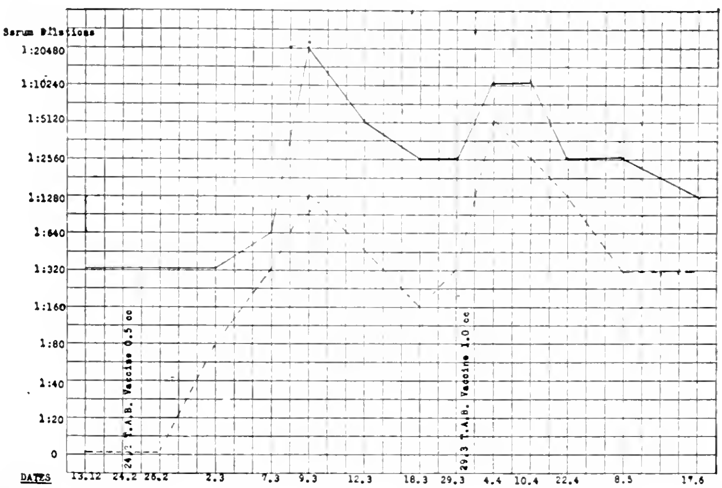


Chart 7.—B. typhosus and Para A agglutinograms in T. A. inoculated person after 2 doses of T. A. B. vaccine.

The Dreyer macroscopic method used with standardized agglutinable cultures not only gives excellent results, but allows comparison to be made of results obtained at different times in different laboratories. These results should be expressed in agglutinin units.

For proper comparison, every step in the method should be standardized; in other words, all workers whose results are to be compared should perform each step in the same way. The most consistent results are obtained by one worker using the same batch of cultures.

Results from different laboratories must be interpreted with caution and allowance made for the personal equation as well as for

possible variations in the cultures. Variations of one or sometimes even two tubes should be disregarded as the changes caused by active infection are much greater than this.

It is recommended that distilled water should be uniformly used as a diluent, and that readings should always be made with a hand glass (4 diameters) in the illumination advised by the Oxford Standard Laboratory.

If standard agglutinable cultures are used, they should be carefully guarded against contamination. If contamination occurs, especially if they have become acid, they should be at once discarded.

In cases in which agglutinations show a marked rise but do not point decisively to any one of the three members of the enteric group, and cultures are negative but clinical evidence suggestive, a diagnosis of "enteric group" should be made. This rise in titer in more than one species is probably due to nonspecific stimulation (i. e., minor or partial agglutinins) or rarely to coagglutination or multiple infection.

We present herewith figures showing the gradual fall in T. A. B. agglutinins in 40 inoculated individuals. If the presence of agglutinins may be taken as an index of protection, these and similar tests show that the methods of prophylactic inoculation now in vogue protect the average individual for more than a year. There is, however, considerable variation in the individual response, and apparently also in the effect of different batches of vaccine.

We also present figures to show that paratyphoid B inoculation in persons who have already received typhoid and para A vaccine, causes a distinct rise in the typhoid agglutinin curve. This fact should be borne in mind in interpreting clinical tests.

A single experiment is given which tends to show that as compared with the American method now in vogue, more efficient prophylactic inoculation may be obtained by giving two (or possible even single) doses at more frequent intervals.

NOTE.—Since this paper was completed an article by Fennel[14] on the same subject has come to hand. The reader is referred to it with the endorsement of the suggestions offered. Various examples of interesting results obtained with the Dreyer method have recently been published by Perry.[15] Other recent investigations by Dreyer and others[16] tend to support our view that prophylactic inoculations would be impaired by lengthening the interval between doses, and giving fewer doses, but repeating the process more frequently.

[14] Jour. Am. Med. Assn., 1918, 70, p. 590.
[15] Lancet, 1918, 1, p. 593.
[16] Ibid., p. 498.

THE ANTIGENIC PROPERTIES OF GELATIN

William A. Starin

From the Department of Pathology, University of Chicago, and the Department of Bacteriology, Ohio State University, Columbus, O.

Notwithstanding the large amount of work given to the subject, the exact character of that group of substances called "antigens" remains obscure. Whether or not chemical analysis will be able to determine this function as a property of any certain chemical group or groups is questionable. Indications seem to point to the idea that physical factors[1]—the molecular size of the substance, the degree of dispersion, the arrangement of certain groups within the molecule—play a part, as well as the strictly chemical composition. As Wells[1] has said, "Whether the chemical differences that determine specificity are of a quantitative nature which can be disclosed by analytic means or whether they are dependent on spatial relationship of the amino-acid radicals, as suggested by Pick,[2] remains to be determined."

The definition of an "antigen" at present is based, not on the chemical composition of the substances but on the peculiar biologic reactions, which make themselves manifest in certain definite ways. It is only as it is capable of fulfilling the conditions of these reactions, that any substance is called an antigen.

Certain general facts, however, are at present fairly well established, as regards antigens. They are proteins, relatively complex in character, with relatively large molecules. The antigenic properties seem to parallel the molecular size and the chemical complexity. The fact cited by Pick that certain types of proteins call forth lytic reactions, while other types call forth agglutination and precipitin reactions, indicates that the function must be dependent on different groups of the molecule. The molecule must evidently possess certain chemical groups in order to call forth certain types of antibodies.

Obermeier, Pick, Vaughan, and others have made the observation that a protein to possess antigenic functions must contain amino-acids

Received for publication March 9, 1918.

1 Chemical Pathology, 191, p. 187.

2 Pick: Biochemie der Antigene, u. s. w. Kolle und Wassermann's Handbuch der Pathogenen Mikroorganismen, 1913, 1, p. 685.

rich in aromatic radicals. According to them, proteins containing none of these radicals serve poorly, if at all, as antigens. This is of peculiar interest in relation to the antigenic properties of gelatin.

Attempts to obtain some knowledge as regards the chemical basis of antigens have been many and varied. They have been conducted by the preparation of definite types of chemical compounds in as pure a state as possible and then studying the antigenic properties of the compounds. Thus, Wells and Osborne[3] have prepared and tested many pure vegetable proteins; Elsesser[4] likewise tested various vegetable proteins for the production of proteolytic ferments; Wells[6] studied various nucleo-proteins; Elliott[7] studied glycoproteins from various sources; Schmidt[8] prepared certain compound proteins, e. g., protamine-edestinate, and studied their antigenic properties; Dale and Hartley[9] tested the antigenic properties of the fractions of horse serum. These are only a few examples indicative of the large amount of work that has been done along this line. An extensive review of these studies is given by Pick.

In view of these studies and particularly because of the peculiar chemical composition of gelatin, the suggestion was made that its antigenic properties be studied.

Gelatin is a rather peculiar protein compound, the exact chemical composition of which is not known. In fact, there is a question whether the term "gelatin" as now used represents a chemical entity. Chemical analyses of gelatin derived from different sources or prepared by different methods, vary considerably.[10] They all agree, however, in the general result, that gelatins are rich in glycocoll, proline, and arginine, but poor or entirely deficient in the amino-acids that contain the aromatic radicals. The study, therefore, of its antigenic properties might be expected to yield some information regarding the relation of the aromatic radicals to antigenic function.

In the literature one can find only a few references to gelatin as an antigen. The first experiments found recorded in the literature are those of Wells,[11] who

[3] Jour. Infect. Dis., 1911, 8, p. 106.
[4] Ibid., 1916, 19, p. 655.
[6] Jour. Biol. Chem., 1916, 28, p. 1.
Ztschr. f. Immunitätsf., O., 1913, 19, p. 598.
[7] Jour. Infect. Dis., 1914, 15, p. 501.
[8] Univ. of Calif. Pub. Path., 1916, 2, p. 157.
[9] Biochem. Jour., 1916, 10, p. 408.
[10] Mathews, A. P.: Physiological Chemistery, 1916, Ed. 2.
Greifenhagen, König u. A. School-Biochem. Ztschr., 1911, 35, p. 217.
Sadikoff: Ztschr. f. Phys. Chemie, 1904, 41, p. 15.
Sadikoff: Ztschr. f. Phys. Chemie, 1903, 39, p. 411.
Plimer: Practical Organic and Bio-Chemistry, 1915, p. 453.
Mörner: Ztschr. f. Phys. Chemie, 1899, 28, p. 471.
Sadikoff: Ztschr. f. Phys. Chemie, 1903, 39, p. 396.
Cohnheim: Chemie der Eweisskörper, 1904, p. 285.
Hoppe-Seyler: Handbuch der Phys. u. Path. Chemischen Analyse, 1909, p. 449.
Allen: Commercial Organic Analysis, 1913, Ed. 4, 8, p. 587.
[11] Jour. Infect. Dis., 1908, 8, p. 88.

was unable to secure anaphylactic reactions with gelatin in guinea-pigs, which lack of anaphylactogenic property he ascribed to the deficiency of aromatic radicals in gelatin. Arthus[12] reports that he was able to sensitize rabbits with gelatin, by intravenous injections. After 5 or 6 injections, he observed lack of absorption of injected material and abscess formation, such as characterizes the reaction of local anaphylaxis (Arthus' phenomenon). Abderhalden[13] reports that the repeated injections of dogs with gelatin are accompanied by the formation of proteolytic ferments, quantitatively specific for gelatin, which may be demonstrated by optical methods. Ascoli and Izar,[14] and Izar[15] report changes in the blood of rabbits repeatedly injected with gelatin which are manifested by changes in the viscosity of the antigen-antibody mixture (meiostagmin reaction). Pick[1] states that gelatin is not capable of stimulating to the production of antibodies. Wells,[16] in discussing anaphylaxis, says, "So far as known, antigens are always protein, and with the *exception* of *gelatin* and a few others, all proteins will produce sensitization in susceptible animals." Pabes and Regazzi[17] sought to produce anaphylactic reactions in rabbits by repeated injections with gelatin, both normal and hydrolyzed, with negative results. They suggest that the cause of the failure is in the character of the gelatin molecule, which statement, of course, means nothing, in the light of our present ignorance of the chemical nature of gelatin.

In view of the conflicting results reported, of the lack of detailed accounts of the experimental methods, and also because in many instances the attempt was made to show only one kind of antibody, it was thought that a more detailed immunological study of gelatin would be worth while.

For this purpose, two kinds of gelatin were used. One was prepared by purification of the best grade of laboratory gelatin obtainable —the French "Gold Leaf." The other type was prepared from the tracheal rings of the beef, giving the product generally called "Cartilage Gelatin," or "Chondrin." The latter was used in a few instances only, serving as specificity control. It has been made the subject of a special investigation, as yet unpublished.[18] Unless specifically noted, the first type of gelatin is that which is referred to in this article.

Since very small traces of proteins are capable of acting as antigens, great care was exercised to avoid accidental contamination with other proteins. The glassware, apparatus, syringes, were all soaked in a strong bichromate-sulphuric acid cleaning mixture for 24–48 hours and then thoroughly washed in cold and hot distilled water. Such apparatus was then used for the gelatin work only. In view of the constant

[12] Arch. Internat. de physiol., 1908, 7, p. 493.
[13] Ztschr. f. Phys. Chemie, 1910, 64, p. 100.
[14] München. med. Wchnschr., 1910, 57, pp. 62, 403, 954, 2129.
[15] Ztschr. f. Immunitätsf., O., 19, 7, p. 189.
[16] Chemical Pathology, 1914, p. 179.
[17] R. acct. Fisiocril, 221, p. 557.
[18] Peeples: Master's Thesis, Ohio State Univ., 1917.

results obtained throughout, it is evident that no foreign proteins, at least capable of acting as antigens, were present.

PREPARATION OF GELATIN

(1) *"Gold Leaf" Gelatin.*—Three separate unbroken samples of this product were obtained. An aliquot part was taken from each sample and the mixture used. The mixture was washed in cold water for 24 hours at refrigerator temperature. It was then dried, and washed in two changes of absolute alcohol for 24 hours. This was followed by washing with ether, two changes, for 24 hours and dried. After drying, the gelatin was dissolved in a minimum amount of warm water. Care was exercised not to have the water hotter than necessary for dissolving, thus attempting to avoid high temperatures which might influence the antigenic properties of the substance. The dissolved gelatin was precipitated by pouring the solution, with constant stirring, into 10 volumes of 95-98% ethyl alcohol. A heavy, white, very tenacious precipitate resulted. This precipitate was allowed to remain in contact with the alcohol for 24 hours or more, when the alcohol was decanted off, and the precipitate redissolved in a minimal amount of warm water. This dissolved the gelatin but left the other alcohol-precipitated material undissolved. This solution was passed through filter paper and reprecipitated into 10 volumes of fresh ethyl alcohol, in which it was again allowed to remain 24 hours. This process of precipitation with alcohol, re-solution in warm water, and filtration, was repeated 4 times. The product following final precipitation was cut up into as fine shreds as possible and washed 24 hours in absolute alcohol in the cold, followed by boiling in absolute alcohol on a water bath, for 20-30 minutes. The alcohol was removed by decantation and the product washed in ether for 24 hours with frequent stirring. This application of ether was repeated, the final ether allowed to evaporate, and the material dried over sulphuric acid in a vacuum desiccator.

The product obtained is hard and horny in consistency—golden to grayish-yellow in color. It is readily soluble in warm water and gels on cooling to 20 C., even in dilutions of 1%.

Application of the precipitation, solubility, and colorimetric tests for gelatin as indicated by Mörner, Sadikoff, Hoppe-Seyler, and Allen gave results which were in conformity to those characteristic of gelatin. According to Berriar[36] gelatin can be separated from other proteins and quantitatively determined by adding to the gelatin solution a mixture of 1 part of picric acid (sat. aq. sol.) plus 4 parts of absolute alcohol At 80 C. all other proteins are precipitated while the gelatin is held in solution. On evaporation of the alcohol following filtration, the gelatin is precipitated quantitatively as cooling progresses. The application of this method to the product obtained gave no indication of any proteins present other than gelatin.

(2) *Cartilage Gelatin (Chondrin).*—For details regarding the preparation of this product, I refer to Allen's Chemical Commercial Organic Analysis. In outline, the method consists in digestion of the tracheal rings of the beef, after removal of all connective tissue, with dilute lime water, under steam pressure, precipitation of the filtered digest with 10-15 volumes of absolute alcohol. Redissolve in water; reprecipitate with acetic acid; filter, neutralize with sodium carbonate; reprecipitate in absolute alcohol, after which the treatment is the same as for the other type of gelatin. The resulting product is a golden brown substance, finely granular, easily soluble in water. It gives the tests regularly ascribed to chondrin.

TOXICITY OF GELATIN

Investigators[19] who have injected animals report that normal gelatin possesses considerable toxicity and thus they experienced considerable difficulty in a series of injections into animals. While no definite attempt was made to determine the lethal dose of the gelatin in this work, the results of a large series of injections would indicate that the toxicity must be relatively low, and that animals tolerate large quantities without any marked permanent ill effects. For example, rabbits were repeatedly injected intravenously, with amounts varying from 0.2-0.4 gm. on each of 3 successive days, without any fatal results. Such injections were, however, accompanied by some loss of weight and by stupor. Intravenous injections of amounts over 0.5 gm. into rabbits resulted fatally, in the few tests made. Neither was there any evidence of any cumulative action. As noted later, many of the rabbits received 10-25 injections, intravenously or intraperitoneally, over a period of 5-10 months without any indications of intoxication.

Guinea-pigs withstood intravenous injections of 0.1 gm. without toxic results being evident.

TYPES OF ANTIBODY REACTIONS STUDIED

The following types of antibody reactions were studied with serum from guinea-pigs, rabbits, and dogs, injected in various ways with gelatin: (a) anaphylaxis; (b) precipitins; (c) production of specific proteolytic ferments; (d) complement binding; (e) meiostagmin reaction.

THE ANAPHYLAXIS REACTION

With the exception of Arthus,[12] who reports positive results in rabbits, the literature reveals that other investigators have obtained negative results in attempting to demonstrate that gelatin is an anaphylactogen. Only very meager reports, however, are obtainable, most authors simply stating that gelatin gave negative results, or that no general symptoms were observed. In carrying out anaphylactic experiments in this investigation, rabbits, guinea-pigs, and dogs were used, and conclusions as regards results were based on (a) general symptoms, (b) variations in temperature following second injection, (c) the character of the blood pressure (in dogs), (d) changes in coagulation time of blood, and (e) necropsy findings.

EXPERIMENTS ON RABBITS

Young rabbits, 700-1,100 gm., were used. The gelatin was dissolved in sterile physiologic salt solution, generally diluted in such a way that 1 c c contained the desired amount for injection. The sensitizing injections were all made intraperitoneally, in doses from 0.04-0.1 gm. The intoxicating dose used was always much larger, being from 5-10 times the sensitizing dose, and

19 Aronson: Personal communication to Dr. H. G. Wells, 1916.
Buglia: Biochem. Ztschr., 1908, 23, p. 215.

injected either intravenously or intraperitoneally. The interval elapsing between the first and second injection was about 16 days. When a third injection was given, this was 10-12 days subsequent to the second. All the usual precautions of cleanliness of apparatus were observed to avoid any possibility of sensitization with other alien proteins.

Results are recorded in accordance with the method suggested by Wells.[20] The same significance is attached to the terms throughout all the anaphylactic work.

TABLE 1

ANAPHYLAXIS EXPERIMENTS WITH GELATIN ON YOUNG RABBITS

Rabbit	Weight, gm.	Sensitizing Injection		Interval, Days	Second Injection		Symptoms	Interval, Days	Third Injection		Symptoms
		Amount, gm.	Mode		Amount, gm.	Mode			Amount, gm.	Mode	
1	785	0.1	INTRAPERITONEAL	16	0.8	Intra-peritoneal	Negative. Slight restlessness	12	0.8	Intra-peritoneal	Negative
2	785	0.04		17	0.2	INTRAVENOUS	Negative	12	0.2	INTRAVENOUS	Same as after injection No. 2
3	775	0.04		16	0.2		Doubtful. Normal in 4 hours	12	0.2		Same as after injection No. 2
4	952	0.04		17	0.4		Doubtful. Slight irritation. Normal in 1½ hours	10	0.4		No change
5	870	0.1		16	0.8	INTRA-PERITONEAL	Negative	10	0.8	INTRA-PERITONEAL	No change
6	1,093	0.1		16	1.0		Doubtful. Temperature fall of 1.8 C.	10	1.0		Negative
7	1,000	...	..	..	1.0		Doubtful. Restless and stupid	12	1.0		Doubtful
8	900	0.05 c c ox serum	Intra-peritoneal	16	1.5 c c ox serum	Intra-venous	Severe. Death in 35 minutes	..			

No. 7—Control rabbit.
No. 8—Control rabbit.

"1. 'Doubtful' Symptoms: The animal scratches itself a few times and perhaps seems a little uneasy, or a trifle ill; the temperature remains normal or falls not more than 1-1.5 C. Such a reaction may occasionally be observed after injections of foreign serum into nonsensitized animals, and is not to be regarded as of any significance.

"2. 'Slight' Symptoms: The animal does not become seriously ill but scratches itself vigorously, is either restless or very somnolent, or both alter-

[20] Jour. Infect. Dis., 1911, 8, p. 88.

nately, hair roughened, eyes usually lacrimate and partially closed. The temperature falls 1-1.5 C. Such a reaction is believed to indicate, usually if not always, a real sensitization and a true anaphylactic intoxication, but it may possibly, sometimes, though rarely, occur from the injection of foreign proteins without sensitization.

"3. 'Moderate' Symptoms: More seriously ill than under 2. Usually the hair becomes rough, with respiratory disturbances and marked lacrimation, fall of temperature from 1.5-3.5 C.; frequently the animal lies down and sometimes there is moderate coughing.

TABLE 2

ANAPHYLAXIS EXPERIMENTS WITH GELATIN ON YOUNG GUINEA-PIGS. SERIES 1

Guinea-pig	Weight, gm.	Sensitizing Injection		Interval, Days	Second Injection		Symptoms	Interval, Days	Third Injection		Symptoms
		Amount, gm.	Mode		Amount, gm.	Mode			Amount, gm.	Mode	
1	283	0.05	INTRAPERITONEAL	18	0.3	INTRAPERITONEAL	Doubtful. Slight restlessness and stupor. Normal in 6 hours*	..	......	..	
2	253	0.05		18	0.5		Negative†	..	......		
3	357	0.05		18	0.3		Negative	10	0.3	INTRAPERITONEAL	Negative
4	426	0.02		18	0.3		Doubtful	10	0.3		Negative
5	329	0.02		18	0.3		Negative	10	0.5		Negative (stupor)
6	352	0.02		18	0.5		Negative	10	0.5		Negative
7	365	0.005		20	0.3		Doubtful	10	0.5		Negative
8	305	0.005		20	0.05	Intracardial	Doubtful	10	0.5		Doubtful
9	293	0.005		20	0.05		Negative	..	......	..	
10	300	0.05	Intracardial	..	......	..	Doubtful				
11	350	0.3	Intraperitoneal				Negative				

* Died 3 days later. No anaphylactic lesions. Pneumonia.
† Died 2 days later. No lesions typical of anaphylaxis. Pneumonia and ascites.
No. 10—Control.
No. 11—Control.

"4. 'Severe' Symptoms: Same as under 3, but so much more marked as to threaten death, but followed by complete recovery in a few hours. In these animals the temperature usually falls 3-4 C. or even more."

It may be noted that in these anaphylactic experiments with gelatin, not much stress can be laid on the relation of stupor to anaphylaxis, since it was found to be a rather common occurrence even among animals receiving the first injection, as also among those animals that had received 5-10 injec-

tions. I believe it is a symptom peculiar to the gelatin, per se, and not related to the phenomenon of anaphylaxis.

Table 1 will serve to summarize the technic and results obtained in the attempt to demonstrate anaphylaxis in rabbits with gelatin.

The results of these experiments indicate that gelatin is not capable of sensitizing young rabbits against subsequent injections of the same material. The "doubtful" symptoms noted in Rabbits 3, 4, and 6 were very mild and no more severe than those observed in control Rabbit 7. The third injection was made for the purpose of seeing whether there was any evidence of the appearance of a refractory stage following the second injection—antianaphylaxis. As will be observed, there was no indication of any changes in the character of the reaction which might be attributed to antianaphylaxis. The rabbits were kept under observation for a period of 10 days following the last injection; no abnormal symptoms were noted.

TABLE 3

Anaphylaxis Experiments with Gelatin on Young Guinea-Pigs. Series 2

Guinea-pig	Weight, gm.	Sensitizing Injection		Interval, Days	Second Injection		Symptoms	Interval, Days	Third Injection		Symptoms
		Amount. gm.	Mode		Amount, gm.	Mode			Amount, gm.	Mode	
1	300	0.01	INTRAPERITONEAL	20	0.075	INTRAJUGULAR	Doubtful	12	0.1	INTRAPERITONEAL	Negative
2	275	0.01		20	0.10		Doubtful	10	0.3		Doubtful
3	290	0.01		20	0.075		Negative	10	0.3		Negative
4	300	0.005		20	0.10		Doubtful	10	0.3		Similar to 2nd injection
5	280	0.005		20	0.075		Doubtful	10	0.3		Doubtful
6	275	0.005		20	0.10		Doubtful	10	0.2		Doubtful
7	300	0.15	Intra-jugular	..	......	..	Doubtful	..	......	.	
8	360	0.15		..	......	..	Doubtful	..	......	..	

No. 7—Control.
No. 8—Control.

Experiments with Young Guinea-Pigs

Three series of tests were made with young guinea-pigs to see whether there could be produced any indication of sensitization against gelatin. Young pigs, averaging about 300 gm. in weight, were used. The pigs were obtained from three entirely different lots, so as to reduce to a minimum any peculiar idiosyncrasies of certain lots. The sensitizing doses were all given intraperitoneally and varied from 0.005-0.05 gm.: One c c of the dissolved gelatin was made to contain the desired amount for injection. The mode of reinjection varied with the different series. Thus, in Series 1 and 3, the material was reinjected intracardially or intraperitoneally. In Series 2, the reinjection was made intrajugularly.

Tables 2, 3, and 4 show in detail the method employed and the results obtained.

The results in all the experiments with guinea-pigs were quite constant. Only a few of the pigs, in which the second injection was made directly into

the jugular vein, was there any evidence whatever of anaphylactic symptoms. Even here the symptoms were so mild as not to indicate a positive reaction, and are to be ascribed, in part, to shock consequent to the anesthetic and the operation exposing the jugular, and in part to the natural primary toxicity of the gelatin. Buglia[20] described a similar series of symptoms in dogs which had received a single intravenous injection of gelatin.

In many cases there was a slight drop in the temperature, but this never exceeded 1.8 C., and was quite transitory, the return to normal being accomplished within 1½ hours, as a maximum.

The coagulation time of the blood, as determined by regular standard methods, showed marked variations. In certain instances an increase of 20-40% in the coagulation time occurred; in other instances there was no change; in still others, an actual decrease. Hence, this must not be considered in reaching conclusions regarding anaphylaxis with gelatin.

TABLE 4

ANAPHYLAXIS EXPERIMENTS WITH GELATIN ON YOUNG GUINEA-PIGS. SERIES 3

Guinea-pig	Weight, gm.	Sensitizing Injection		Interval, Days	Second Injection		Symptoms	Interval, Days	Third Injection		Symptoms
		Amount, gm.	Mode		Amount, gm.	Mode			Amount, gm.	Mode	
1	300	0.001	Intraperitoneal	15	0.23	Intracardial	Negative				
2	280	0.001	Intraperitoneal	15	0.20	Intracardial	Doubtful	8	0.20	Intraperitoneal	Negative
3	250	0.005	Intraperitoneal	15	0.25	Intracardial	Negative	8	0.3	Intraperitoneal	Negative
4	300	0.005	Intraperitoneal	15	0.25	Intracardial	Negative				
5	250	0.005	Intraperitoneal	15	0.20	Intracardial	Negative	8	0.3	Intraperitoneal	Negative
6	275	0.005	Intraperitoneal	15	0.20	Intracardial	Doubtful				
7	300	0.25	Intracardial	..	......	Intracardial	Negative				
8	290	0.25	Intracardial	..	......	Intracardial	Doubtful				

No. 7—Control.
No. 8—Control.

Controls were similarly treated throughout, and the symptoms presented were practically identical with those in the animals presumably sensitized.

Only 2 of the pigs tested died—Nos. 1 and 2, Series 1. In neither case were the anatomic findings those recognized as characteristic of death from anaphylactic shock.

In none of the animals subjected to a third injection was there any evidence of a condition of increased refractiveness, simulating antianaphylaxis. The symptoms following the third injection could not be differentiated from those following the second.

Considering all factors involved and the results obtained in the series of 27 guinea-pigs, one must conclude that gelatin is not capable of functioning as an antigen, as expressed by anaphylactic reactions in young guinea-pigs, corroborating the observation of Wells.[21]

EXPERIMENTS WITH YOUNG DOGS

To determine whether gelatin is capable of sensitizing dogs to subsequent injections of the homologous material, 2 young normal dogs, weighing respec-

[21] Jour. Am. Med. Assn., 1908, 50, p. 527.

tively 3.8 kg. and 4.7 kg. were injected intraperitoneally with gelatin; the one receiving 0.1 gm., the other 0.5 gm. gelatin dissolved in sterile physiologic salt solution so as to make a 5% solution. The dogs showed no reaction whatever to these preliminary injections of gelatin.

After an interval of 15 days, they received a second injection. The dose on reinjection was 2 gm. of gelatin for the smaller dog, and 3 gm. for the larger. The total volume of material injected was 10 c c and 12 c c, respectively. The second injection was made into the femoral vein.

Blood pressure tests prior to the second injection and subsequent to it showed no fall more marked than that exhibited by a normal dog during an interval of 45 minutes. Necropsy failed to show any of the anatomic findings characteristic of death due to anaphylaxis.[5] No changes were observed in the coagulation time of the blood.

TABLE 5

ANAPHYLAXIS EXPERIMENTS WITH GELATIN ON YOUNG DOGS

Dog	Weight, gm.	Sensitizing Injection		Interval, Days	Second Injection		Results				
							Blood Pressure	Coagulation Period		General Symptoms	Necropsy
		Amount, gm.	Mode		Amount, gm.	Mode		Pre-injection	Post-injection		
1	3,800	0.1	Intraperitoneal	15	2.0	Intravenous	The difference in blood pressure before and after injection did not vary 10 mm. of Hg. during an interval of 45 minutes observation	2′30″	2′35″	Negative	Negative
2	4,700	0.5	Intraperitoneal	15	3.0	Intravenous		3′15″	3′25″	Negative	Negative
3	4,500	......	..	..	3.0	Intravenous	No change in pressure following injection greater than 10 mm.	2′40″	2′50″	Negative	Negative

These consistently negative results are in accord with those of Aronsen, who attempted to sensitize dogs against gelatin.

THE PRECIPITIN REACTION

In order to study the precipitin reaction, young normal rabbits were used. Various modes of injection were employed. In one series the intensive method of immunization, first advocated by Fornet and Miller,[22] and later by others[23] was used. This consists of daily intraperitoneal injections of the antigen. In some cases slower methods, consisting of intravenous injections at intervals of about 5 days, were employed. In still other cases, a combination of the two methods previously mentioned were used. Two or three injections were given the rabbit by one of the above methods, and after an interval of 10-12 days the animal was bled and tests made. Following a rest period, which varied

[22] Ztschr. f. biol. Technik u. Methodik, 1908, 1, p. 201.

[23] Bonhoff and Tszuki: Ztschr. f. Immunitätsf., 1908, 1, p. 201.

from a few days to a month, the animals were reinjected in a similar manner. Many of the rabbits used received from 0.6-12.0 gm. of gelatin during the entire period of treatment. In certain instances this period of treatment was as long as 9 months.

The dosage of the gelatin used per injection varied, of course, with the method employed. The details as regards the amounts of gelatin, periods of injections, etc., will be found in Tables 6 and 7. The gelatin was dissolved in sterile physiologic salt in such dilutions as to make the solution 0.5% gelatin. The injections of the gelatin were well tolerated by the rabbits. Only slight loss of weight, which was temporary, and slight stupor were observed.

The blood was obtained in the usual manner by bleeding from the posterior auricular vein, and was allowed to clot spontaneously. The serum was removed

TABLE 6

Summary of Injections of Rabbits with Gelatin for Antibody Reactions

Rabbits	Injections: Groups of Injections	Injections: Total No. Injections	Injections: Mode	Injections: Total Gelatin, gm.	Injections: Interval of Injection	No. Tests	Time of Precipitin Tests
1	6	17	Intraperitoneal	12	July 23, 1916-Dec. 3, 1916	6	Aug. 4, 1916-Dec. 18, 1916
2	5	14	Intraperitoneal	12	July 23, 1916-Dec. 1, 1916	5	Aug. 4, 1916-Dec. 9, 1916
3	9	25	Intraperitoneal Intravenous	9.8 5.0	July 23, 1916-May 1, 1917	11	Aug. 4, 1916-May 15, 1917
4	5	12	Intravenous	2.0	Nov. 19, 1916-Mar. 24, 1917	4	Dec. 1, 1916-Apr. 4, 1917
5	2	6	Intravenous	1.8	Aug. 8, 1916-Oct. 1, 1916	3	Aug. 23, 1916-Oct. 12, 1916
6	5	10	Intravenous	1.5	Nov. 19, 1916-Apr. 21, 1917	4	Dec. 3, 1916-May 2, 1917
7	4	10	Intravenous	1.6	Nov. 19, 1916-Aug. 21, 1917	2	Dec. 5, 1916-May 2, 1917
8	1	3	Intravenous	0.8	May 26-29, 1917	2	June 6 and June 9, 1917
9	1	3	Intravenous	0.8	May 26-29, 1917	2	June 6 and June 9, 1917
10	1	3	Intravenous	0.8	May 26-29, 1917	2	June 6 and June 9, 1917
11	1	3	Intravenous	0.8	May 26-29, 1917	2	June 6 and June 9, 1917

and kept on ice until used. Tests were always made within 24 hours after drawing. While precipitins do not disappear rapidly from a serum on standing, the effort was always made to use relatively fresh serum.

In the tests, the general procedure was to make various dilutions of the antigen (gelatin) in sterile physiologic salt solution and filter until perfectly free from suspended matter; the filtrate was placed in clean tubes, 1.5 c c in each tube. By means of a capillary pipet, 0.1 c c of the serum was layered beneath the gelatin. In a few instances, the serum was diluted and the gelatin kept constant. Readings were made after 20-30 minutes at room temperature after 2 hours at 37 C., and again after remaining at refrigerator temperature over night.

A total of 11 rabbits were used in the determination of the precipitin reaction, many being tested several times. Tables 6 and 7 show the distribution of the tests on the different animals used, the details of the immunization and the tests.

All tests were made according to this plan—all uniformly negative. As controls, tests were made with serum from rabbits injected with various other proteins, e. g., egg albumin, sheep serum, etc., the customary precipitin reactions here appearing very definitely.

These results indicate that in rabbits gelatin does not possess the property of stimulating the production of specific precipitins when introduced as described.

COMPLEMENT FIXATION

Since complement fixation is, in general, one of the most delicate tests for the detection of specific antibodies, a series of such tests were made, using serum of the same rabbits as for precipitin reactions. The plan was to make all the various tests at each drawing of the blood. The total number of complement fixation tests was smaller, however. The details follow:

TABLE 7

The following protocol of a single test will serve to illustrate the details of the precipitin tests:

PRECIPITIN TEST

Tube	A. Serum Constant, 0.1 c c Gelatin Variable				B. Gelatin Constant, 0.02 gm. Serum Variable			
	Amount of Gelatin gm.	Results			Serum Dilution	Results		
		30 min.	2 hrs.	12 hrs.		30 min.	2 hrs.	12 hrs.
1	1 c c = .05	0	0	0	1.4 c c of 1/10	0	0	0
2	.04	0	0	0	1/20	0	0	0
3	.02	0	0	0	1/50	0	0	0
4	.01	0	0	0	1/100	0	0	0
5	.005	0	0	0	1/300	0	0	0
6	.003	0	0	0	1/600	0	0	0
7	.002	0	0	0	1/1,000	0	0	0
8	.001	0	0	0	1/2,000	0	0	0
9	.0005	0	0	0	1/5,000	0	0	0
10	.05 (no serum)	0	0	0	1/10 + salt	0	0	0
11	1.4 c c salt (control)	0	0	0	No serum, salt + gelatin	0	0	0

Gelatin is expressed in gm. per tube; total volume, 1.5 c c.

"Antigenic" Solutions.—The gelatin was dissolved in physiological salt solution and then standardized. This consisted in determining the normal anticomplementary value of the gelatin, and its nonspecific hemolytic properties. The anticomplementary value as determined a number of times was quite constant. The nonspecific hemolytic action did not manifest itself until quantities much larger than the anticomplementary value were used. In actual tests not more than 1/3 to 1/2 the anticomplementary value was used.

Hemolytic amboceptor was the serum of rabbits injected with sheep corpuscles. The serum was capable of completely hemolyzing 1 unit volume of erythrocytes in dilutions of 1:3000. In actual testing, 2 such units of amboceptors were used.

A 3% suspension of well washed sheep corpuscles was used.

Fresh guinea-pig serum served as complement; 1½ times the standard unit was used.

The serum from the rabbits was inactivated by heating to 56 C. for 30 minutes.

In the fixation tests, two methods were used. The one most extensively employed was the original method of Bordet in which a constant quantity of the properly standardized antigen is used and varying amounts of serum. The other method, used in a relatively few instances, was that suggested by Kilgore and Myers,[24] and others, in which the amount of antigen is varied, and the serum kept constant.

The antigen, inactivated serum, and complement were mixed in the order named and incubated 1 hour at 37 C., when the hemolytic system was added. The mixture was then incubated 1½ hours at 37 C. Readings were made immediately after incubation and again after remaining in a refrigerator over night. The usual control tests were always made.

A total of 29 tests by the original Bordet method were made using serum from 11 different rabbits. A total of 10 tests were made by the second method.

The following protocols will illustrate the details of the procedure:

TABLE 8

COMPLEMENT FIXATION. GELATIN (ANTIGEN) CONSTANT: SERUM VARIABLE

Tube	Gelatin	Serum	Guinea-Pig Serum Complement	Salt		Hemolytic System		Results	
						Amboceptor	Erythrocytes	After 1½ Hrs. Incubation	After 12 Hrs. on Ice
1	½ c c = .002 gm.	0.1 c c	0.4 c c	To make volume equal 1½ c c	Incubate 1 hr. at 37 C.	0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
2	½ c c = .002 gm.	0.05 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
3	½ c c = .002 gm.	0.02 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
4	½ c c = .002 gm.	0.01 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
5	½ c c = .002 gm.	0.005 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
6	½ c c = .002 gm.	0.000 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
7	0	0.1 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
8	0	0	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
9	0	0	0			0.3 c c	0.3 c c	No hemolysis	No hemolysis
10	0	0	0.4 c c			0	0.3 c c	No hemolysis	No hemolysis
11	0	0	0			0	0.3 c c	No hemolysis	No hemolysis

Anticomplementary volume of gelatin = 0.006 gm.

The results were constantly negative throughout the entire series. There is no indication of the presence of any substance in the serum of injected animals, which in the presence of "antigen" (gelatin) is capable of binding complement. Thus, as in the case of precipitins, gelatin does not exercise any antigenic functions in the bodies of young rabbits that make themselves evident by the binding of complement.

THE PRODUCTION OF SPECIFIC PROTEOLYTIC FERMENTS

The introduction of alien proteins into the body of an animal, parenterally, is now generally considered to result in the production of an excess of ferment

[24] Arch. Int. Med., 1916, 19, p. 293.

capable of breaking down and destroying the material introduced. The exact nature of the process, and the character of the ferments are still open to discussion. Whether there is actual production[25] of specific ferments, whether it is a process of mobilization of nonspecific ferments, or whether it is an adsorption[26] of normal antiferments, permitting the action of nonspecific normal ferments, are questions that cannot be considered settled.

According to Vaughan[27] and his school, these ferments are specific and manifest themselves in vivo, with a digestion of the specific material on reinjection—thus explaining anaphylaxis as an intoxication due to toxic protein products. Assuming the truth of this view, it is evident that gelatin did not give any indications of the production of ferments, as shown in the series of anaphylactic tests.

TABLE 9

COMPLEMENT FIXATION. GELATIN (ANTIGEN) VARIABLE: SERUM CONSTANT

Tube	Gelatin	Serum	Guinea-Pig Serum Complement	Salt		Hemolytic System		Results	
						Amboceptor	Erythrocytes	After 1½ Hrs. Incubation	After 12 Hrs. on Ice
1	0.0012 gm.	0.1 c c	0.4 c c	To make volume equal 1½ c c	Incubate 1 hr. at 37 C.	0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
2	0.0012 gm.	0.2 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
3	0.002 gm.	0.1 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
4	0.002 gm.	0.2 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
5	0.003 gm.	0.1 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
6	0.003 gm.	0.2 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
7	0.003 gm.	0.0 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
8	....	0.2 c c	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
9	....	......	0.4 c c			0.3 c c	0.3 c c	Complete hemolysis	Complete hemolysis
10	....	......	0			0.3 c c	0.3 c c	No hemolysis	No hemolysis
11	....	......	0.4 c c			0	0.3 c c	No hemolysis	No hemolysis
12	....	......	0			0	0.3 c c	No hemolysis	No hemolysis

According to Abderhalden, it is possible to demonstrate the presence of these ferments in vitro.

The mixing of a specific antiserum with its antigen results in a cleavage process made manifest by various means, chief of which are changes in the dialyzable character of the antigen, and differences in the way they rotate a beam of polarized light.

Abderhalden reports that he was able to detect such ferments in the serum of dogs, injected with gelatin, as shown by changes in rotation of light. The extent of the change was slight and not strictly specific.

[25] Abderhalden: Defensive Ferments of the Animal Organism, trans. by J. O. Gavronsky and W. F. Lanchester, 1914.

[26] Jobling and Peterson: Jour. Exper. Med., 1914-16 (V. 19-22).
Bronfenbrenner: Ibid., 1913, 18, p. 221.

[27] Vaughan: Protein Split Products in Relation to Immunity and Disease, 1913.

The same rabbits previously mentioned, and a few new ones not subjected to prolonged treatment, were used to see whether any evidence of such proteolytic ferments could be detected with the gelatin as prepared for these experiments. The rabbits were generally tested within 5 days after the last injection; both the "dialysis" method and the "polariscopic" method were used. The greater number of tests were by the first method.

A. Dialysis Method. The Substrate.—The gelatin was dissolved in sterile distilled water. The solution varied in different tests from 1-3%. This material was freshly prepared just before each test and carefully tested for the presence of any diffusible substances normally present.

The Dialyzing Sacs: Two types of dialyzing sacs were used—Schleicher and Shull's parchment sacs, No. 579a, and sacs prepared from celloidin. Parallel tests were carried out with the two kinds of sacs.

All sacs were carefully standardized prior to being used; they were subjected to the following tests.

(a) Impermeability to gelatin solutions of the strength used.

(b) Impermeability to complex proteins, e. g., egg albumin, and rabbit serum.

(c) Permeability to peptones and proteoses as shown by the diffusibility of solutions of Witte's peptone.

The Serum: As has been observed by many, and particularly emphasized by Abderhalden,[25] the character of the serum is of primary importance. Great care was exercised therefore in collecting it. Rabbits were bled only after a period of fasting, since it has been shown that serum drawn shortly after meals frequently contains dialyzable products. The blood was drawn from the ear vein, and by using care could be obtained free from visible hemoglobin. Blood tinged with hemoglobin was discarded. No attempt was made to demonstrate hemoglobin spectroscopically, but controls were used to check up any normal dialyzable products. In a few instances the serum obtained was subjected to a predialysis, using as an agent physiologic salt solution, for a period varying from 6-24 hours. Prolonged dialysis was found unsatisfactory since it robbed the serum of any ferment action, as shown by controls. The results obtained with predialyzed serum did not vary much from those obtained when undialyzed serum was used. All serums were used within 4-6 hours after drawing the blood.

The Test: This was carried out by placing 1 c c of the gelatin solution and 0.5 c c of the serum to be tested into well cleaned and standardized sacs. The outer surface of the sacs was carefully washed in running distilled water to remove any possible protein contamination, and then placed into vessels containing 10 c c of sterile distilled water. The material within the sac and the water within the vessel were covered with a layer of toluene. The vessels were then covered with close fitting glass covers. The tests were always in duplicate, using both a parchment and a celloidin sac for each test. The sacs were incubated either at room temperature for 24 hours, or at 37 C. for 16 hours. The attempt was made to maintain as uniform conditions as possible. After the period of incubation, 5 c c of the dialysate were removed to a perfectly cleaned tube, and tested for the presence of diffusible proteins as directed by Abderhalden,[25] using ninhydrin throughout. The boiling time was definite and constant, being one minute in all cases.

The depth of color obtained is indicated by the usual "+" signs. Thus "+ + + +" indicates a color of maximum intensity, while "+ + +," "+ +,"

"+," and "O" indicate colors of decreasing intensity to complete absence. Readings of all tests were made 40 minutes after the cessation of boiling, and in good white light.

The following control tests were made and the results compared with those of the test proper. (a) Normal rabbit serum vs. gelatin; (b) anti-ox-serum vs. gelatin; (c) anti-coli-serum vs. gelatin; (d) anti-chondrin serum vs. gelatin: (e) anti-egg albumin serum vs. gelatin, and (f) anti-gelatin serum vs. chondrin.

Table 10 is a composite protocol indicating the character of the tests and the results. The serum of 9 rabbits injected with gelatin was used in 18 tests, 15 of which were with undialyzed serum and 3 with dialyzed.

TABLE 10

PROTEOLYTIC FERMENTS. SERUM NOT PREDIALYZED

Tube	Substrate	Serum	Results With Rabbit					
			No. 1		No. 2		No. 5	
			10/14	12/11	10/14	12/11	10/15	4/16
1	1 c c gelatin 1-5%	0.5 c c antigelatin	+++	tr	+++	++	++	0
2	1 c c gelatin 1-5%	0.5 c c antigelatin	+++	+	+++	++	+++	+
3	1 c c gelatin	0.5 c c physiological salt	..	..	..	..	..	..
4	1 c c physiological salt	0.5 c c antigelatin	+	0	tr	0	+	0
5	1 c c chondrin	0.5 c c antigelatin	+	+	+	0	+	0
6	1 c c chondrin	0.5 c c antigelatin	0	tr	+	+	+	0
7	1 c c gelatin	0.5 c c antiprecipitin	..	..	..	..	..	..
8	1 c c gelatin	0.5 c c antiprecipitin	..	..	..	..	..	..
9	1 c c gelatin	0.5 c c anticoli	..	..	..	..	..	..
10	1 c c gelatin	0.5 c c anticoli	..	..	..	..	..	..
11	1 c c gelatin	0.5 c c normal	..	..	..	..	..	..
12	1 c c gelatin	0.5 c c normal	..	..	..	..	..	..
13	1 c c physiological salt	0.5 c c normal	..	..	..	..	..	..
14	1 c c physiological salt	0.5 c c anticoli	..	..	..	..	..	..
15	1 c c physiological salt	0.5 c c antiprecipitin	..	..	..	..	..	..
16	1 c c gelatin	0.5 c c chondrin	..	..	..	..	..	..

Proteolytic Ferments. Serum Predialyzed

							4/16	4/17
1	1 c c gelatin 1-5%	0.5 c c antigelatin	..	..	..	..	+++	+++
2	1 c c gelatin	0.5 c c antigelatin	..	..	..	..	+++	+++
3	1 c c salt solution	0.5 c c antigelatin	..	..	..	..	+	+
4	1 c c chondrin	0.5 c c antigelatin	..	..	..	..	++	++
5	1 c c chondrin	0.5 c c antigelatin	..	..	..	..	++	++

Tubes 7 and 8 represent a precipitin serum. In a part of the tests this was an antisheep serum, active against its specific antigen in a dilution of 1 : 10,000; in another group it was an egg albumin serum with a specific activity of 1 : 15,000.

Tubes 9 and 10 represent an anti-coli-serum which agglutinated its homologous antigen in dilutions ranging from 1 : 1000 to 1 : 1500.

The results tabulated seem to indicate that the injection of the rabbits with gelatin has resulted in the increased presence of proteolytic ferments. The results, however, are neither uniform nor specific. Thus Rabbits 1, 3, and 5 show periods when these ferments are quite marked, while at other times they are practically lacking. The lack of specificity is observed in the presence of dialyzable material in Tubes 7 and 8, 9 and 10, and 16. At most, the results would seem to indicate a quantitative specificity and this is neither marked nor

constant. These results agree in the main with those of Elsesser[4] as regards the specificity of proteolytic ferments as revealed by the dialyzing technic of Abderhalden.

Serum, predialyzed, did not yield any more conclusive results than undialyzed serum.

No difference could be noted in the efficiency of the celloidin and parchment sacs, when properly standardized. Sac 1 in duplicate is a Schleicher and Shull 579a, and 2 a celloidin sac.

B. Optical Method—Since Abderhalden's report on an antigelatin ferment is based on the polariscopic method, 2 rabbits were injected intravenously with gelatin and tested according to this method.

TABLE 10—*Continued*

Proteolytic Ferments. Serum not Predialyzed

Results With Rabbit									Controls						
No. 7		No. 9	No. 10	No. 11	No. 12	No. 3									
12/11	4/16	6/13	6/13	6/13	6/13	10/14	12/11	14-16	10/14	10/15	12/11	4/16	6/13	6/17	4/16
+++	+++	+++	0	++	+	+++	++	0							
+++	+++	++	+	+	+	+++	+++	+							
..	..	..	..	..	..	..	..	..	0	0	0	+	+	+	0
+	+	+	+	0	tr	0	tr	0							
++	+	++	+	+	++	++	+++	+							
+	+	+	+	tr	tr	++	++	0							
..	..	..	..	..	..	..	..	..	tr	+	+	+++	+	+	++
..	..	..	..	..	..	..	..	..	tr	+	+	++	0	+	++
..	..	..	..	..	..	..	..	..	+	++	+	+	0	0	tr
..	..	..	..	..	..	..	..	..	+	++	+	+	tr	0	+
..	..	..	..	..	..	..	..	..	0	+	0	+	tr	0	+
..	..	..	..	..	..	..	..	..	0	+	tr	0	0	tr	+
..	..	..	..	..	..	..	..	..	tr	tr	0	0	tr	tr	tr
..	..	..	..	..	..	..	..	..	0	0	tr	0	0	+	+
..	..	..	..	..	..	..	..	..	0	0	0	0	0	tr	0
..	..	..	..	..	..	..	..	..	..	..	..	+++	++	++	++
Proteolytic Ferments. Serum Predialyzed															
				6/17			4/16	4/17							
..	..	++	++	..	..	..	+++	+++							
..	..	++	+	..	..	..	+++	+++							
..	..	tr	+	..	..	..	+	0							
..	.	++	tr	..	..	..	++	tr							
..	..	+	0	..	..	..	+	tr							

These rabbits received a total of 1.0 gm. of gelatin in three groups of injections between June 25 and Aug. 9, 1917. One rabbit received a total of 7 injections; the other received 6 injections.

The method outlined by Abderhalden was followed and the tests made 5 days subsequent to the last injection. Fresh serum, drawn only a few hours prior to testing was used. Serum from a normal rabbit was used as a control.

The basis for recording the results as negative is the fact that the variations in the degree of rotation of the polarized light were no greater when using the serums from treated animals than when the normal serum was used. Neither was the reaction specific, as shown by similar results being obtained when testing the serums with the homologous substrate, the heterologous substrate, or the salt solution control.

While the number of tests carried out was very limited, the general results gave no indications that a specific proteolytic ferment had been produced by repeated injections of gelatin into rabbits.

MEIOSTAGMIN REACTION

Ascoli and Izar attempted to show that the production of specific antibodies, as a result of injections, could be made evident by certain physicochemical methods dependent on the changes in the viscosity resulting from the interaction between the serum and the antigen. Many others since that time have reported results with this method but with lack of uniformity. Izar[24] in a later publication reports positive results when serum from rabbits injected with gelatin was tested in this way. No attempt is made to establish the efficiency of the meiostagmin reaction in this work, but simply to duplicate the work of Izar with the hope that it might contribute something to the question of the antigenic properties of gelatin.

A series of rabbits consisting of Nos. 2, 3, 5, and 7 used in other tests, with 2 rabbits injected with chondrin and 2 normal rabbits, were used.

TABLE 11

POLARISCOPIC METHOD EMPLOYED

Test	Rabbit	Amount Gelatin Injected Intravenously	Interval of Injections	Total No. Injections	Substrate	Amount of Serum	Results
1	(a)	1 gram	June 25 to Aug. 9, 1917	7	Gelatin 1%	1½ c c	Negative
	(a)				Albumin 1%	1½ c c	Negative
	(a)				Salt solution	1½ c c	Negative
2	(b)	1 gram		6	Gelatin 1%	1½ c c	Negative
	(b)				Albumin 1%	1½ c c	Negative
	(b)				Salt solution	1½ c c	Negative
3	(c)	Normal rabbit		0	Gelatin 1%	1½ c c	Negative
	(c)				Albumin 1%	1½ c c	Negative
	(c)				Salt solution	1½ c c	Negative

One series of experiments was carried out following the general method discussed by Ascoli and Izar; the other series by a slightly different method described by Izar. The principles involved in both are alike, being simply a difference in details.

The indication of a positive reaction is a decrease in the surface tension of the solution with an increase in the number of drops in a unit volume of the antigen-antibody mixture. Also a difference in the rate of delivery when the mixture is discharged from a special capillary pipet. In this work a Traube stalagmometer was used.

Technic. First Method.—The instrument was carefully cleaned with water followed by several washings with absolute alcohol and ether.

This process of cleaning was repeated after each test. The instrument was finally rinsed in the fluid to be tested. To insure uniformity of temperature, which is an important factor in viscosity tests, the instruments were placed inside an incubator, and tests conducted in the closed chamber. The variations in temperature did not exceed 2½ C. The incubator, a large heavy one, was

placed on a cement floor in a basement to eliminate any confusing vibratory factors.

The serum was obtained 5 days subsequent to the last injection and used within 10 hours after drawing the blood. The serum was diluted with equal volumes of physiologic salt solution. The gelatin was similarly diluted 1:1000. In the test, 9 c c of the diluted serum was thoroughly mixed with 1 c c of the diluted gelatin, and the number of drops per unit volume immediately determined. The mixture was then incubated 1 hour at 37 C. and the

TABLE 12

SERIES A. MEIOSTAGMIN REACTION WITH GELATIN VS. RABBIT SERUM

No. Rabbit	Serum	Antigen	Results. No. Drops per Unit Volume					
			Preincubation			Postincubation, 1 Hour at 37 C.		
2	9 c c–1:1	1 c c gelatin 1:100	64.1	64.0	64.2	64.3	64.2	64.0
2	9 c c–1:1	1 c c chondrin 1:100	63.4	63.6	63.4	63.5	64.1	63.8
3	9 c c–1:1	1 c c gelatin 1:100	63.7	63.5	63.8	62.8	63.4	62.9
3	9 c c–1:1	1 c c chondrin 1:100	62.8	62.7	63.0	62.9	63.0	63.1
5	9 c c–1:1	1 c c gelatin 1:100	62.9	63.5	63.2	63.1	63.6	63.2
5	9 c c–1:1	1 c c chondrin 1:100	63.0	63.1	62.8	63.4	63.2	63.0
7	9 c c–1:1	1 c c gelatin 1:100	64.3	64.1	64.4	64.0	64.2	64.1
7	9 c c–1:1	1 c c chondrin 1:100	63.6	63.8	63.6	63.8	64.0	64.0
Normal	9 c c–1:1	1 c c gelatin 1:100	62.4	62.9	62.8	62.3	62.7	62.9
Normal	9 c c–1:1	1 c c gelatin 1:100	62.8	62.7	62.9	63.1	62.6	63.0

TABLE 13

MEIOSTAGMIN REACTIONS

No. Test	Nature of Mixture	Results—Rabbit No. 1				Results—Rabbit No. 2			
		Time of Discharge		No. Drops		Time of Discharge		No. Drops	
		Pre-incubation	Post-incubation	Pre-incubation	Post-incubation	Pre-incubation	Post-incubation	Pre-incubation	Post-incubation
a	4.5 c c immunized rabbit	3′45″	3′50″	64.1	65.2	3′30″	3′25″	63.1	63.0
	serum+5.5 c c of 0.85%	3′50″	3′40″	63.6	54.2	3′40″	3′30″	63.4	63.1
	NaCl	3′50″	3′45″	64.0	64.0	3′40″	3′25″	63.2	63.1
b	4.5 c c immunized rabbit	4′25″	4′10″	63.4	63.1	3′40″	3′35″	63.2	64.3
	serum+4.5 c c NaCl+	4′25″	4′20″	63.6	63.2	3′40″	3′40″	63.1	63.4
	1 c c 1% gelatin sol.	4′25″	4′20″	63.4	63.6	3′35″	3′35″	63.1	63.1
c	1 c c immunized rabbit	3′00″	3′00″	60.1	61.0	3′05″	3′05″	60.1	60.0
	serum+9 c c of 0.85%	3′00″	3′00″	60.1	60.2	3′05″	3′05″	60.0	60.5
	of NaCl	3′05″	3′00″	60.3	60.3	3′10″	3′05″	60.9	60.3
d	1 c c immunized rabbit	3′05″	3′20″	60.9	61.5	3′15″	3′10″	60.0	61.0
	serum+8 c c NaCl+1 c c	3′10″	3′20″	61.0	61.2	3′20″	3′15″	60.5	60.2
	of 1% gelatin solution	3′10″	3′20″	61.1	61.4	3′15″	3′15″	60.1	60.1
e	9 c c of 0.85% NaCl+1 c c	3′10″	3′20″	62.1	62.2				
	of 1% gelatin solution	3′15″	3′25″	61.5	62.0				
		3′10″	3′15″	62.0	62.1				
f	4.5 c c normal rabbit	3′55″	4′10″	63.2	63.0				
	serum+4.5 c c NaCl+	4′20″	3′55″	63.2	63.4				
	1 c c gelatin solution	4′15″	3′50″	63.1	63.2				
g	1 c c normal rabbit	3′05″	3′10″	60.1	60.3				
	serum+8 c c of 0.85%	3′05″	3′05″	60.0	60.2				
	NaCl+1 c c gelatin sol.	3′10″	3′10″	60.5	60.1				

number of drops per unit volume again determined. Serum from normal rabbits and from rabbits injected with chondrin was similarly tested as controls. Table 12 shows the results obtained by this method.

Technic—Second Method.—Two rabbits were used in this series. They received a total of 0.8 gm. of gelatin intravenously on 3 successive days. The test was made on the 7th day after the last injection. The technique was that of Izar,[24] in his work on gelatin. In this test not only the number of drops per unit volume was determined, but also the rate of discharge. Incubation time was 1 hour at 37 C.

As a result of their work, Ascoli and Izar came to the conclusion that only differences greater than 3 drops, per unit volume, between the preincubation and the postincubation tests constitute a positive result. Taking this as a standard it is evident that in neither of the series here reported is there any indication of a positive reaction. The maximum difference is less than 1.5 drops and even this is not constant.

Thus the results are not in accord with those of Izar,[24] but seem to show that gelatin injected into rabbits does not stimulate the production of antibodies, as shown by changes in viscosity of such a gelatin-antiserum mixture.

SUMMARY

A study of the results of anaphylactic, precipitin, complement-binding, and meiostagmin reactions, shows that all were constantly negative, with gelatin as an antigen, in rabbits, guinea-pigs, and dogs, the recipients of the injections.

The results when the serums of such animals were tested for the presence of increased proteolytic action are somewhat variable and indefinite. There are some indications that there had been an increase in the ferments active against gelatin, but this is not constant nor marked. The action is not strictly specific since considerable digestion takes place with other substrates. At most, one can say it may be quantitatively specific.

Gelatin as prepared and used in this series of experiments has only a slight toxicity for guinea-pigs, dogs, and rabbits. Such animals tolerate relatively large doses, even when the injections are made intravenously. Neither is there any evidence of any cumulative action of the toxicity of the gelatin, since many of the animals received large quantities over long intervals of time.

No attempt has been made to determine what might be responsible for this evident lack of antigenic property exhibited by gelatin. Whether this is due to the lack of amino-acids containing aromatic radicals, to peculiar molecular characteristics, or to some other factor, is not known. The fact established is, that in the types of animals used and with the tests employed, no antibodies could be detected as the result of antigenic action of gelatin.

THE EFFECT OF EXPOSURE TO COLD ON ANTIBODY PRODUCTION

Alvin George Foord

From the Department of Pathology of the University of Chicago, and the Otho S. A. Sprague Memorial Institute, Chicago.

While it is a generally accepted belief that cold is an etiologic factor in the production of disease, especially of the respiratory tract, its effect on antibody production and leukocytic activity has not been studied thoroughly. Numerous experiments have been made to determine the anatomic and pathologic changes produced by cold without the intervention of infectious organisms, and much more experimental work has been done on the effect of cold on the susceptibility to bacterial and protozoon infections, but in most of the latter group of experiments no analyses of the antibody production have been made, mainly because of the fact that most of this work was done before immunology reached its present position. Brief mention of the results obtained by various observers may be made here, the summary of the literature including the results of chilling experiments for other purposes than to determine the influence of cold on the production of immune bodies.

In regard to the anatomic and pathologic changes produced by exposure to cold per se, the work of Walther,[1] in 1862, is perhaps the first. He showed that chilling rabbits until their rectal temperature had decreased 18-20 C. caused a suppression of their secretions, and, if the animals died, hyperemia and edema of the lungs and air passages and a hydrothorax similar to pleurisy with effusion were found. Lassar[2] demonstrated that chilling caused diarrhea in many cases and an albuminuria with hyaline casts 1 to 2 days after the chilling; microscopically the kidneys gave a constant picture of interstitial inflammation. Myer, Lierheim and Siegel[3] caused albuminuria and hematuria by immersing dogs in cold water. Sections of the kidneys showed cloudy swelling, hyaline and necrotic patches and hemorrhage. Preble[4] obtained similar results, using rabbits, and he also found that pleural and pericardial hemorrhages were frequent in animals chilled to death. Aufrecht[5] reports experiments on 3 rabbits in which he thinks he has shown that chilling causes thrombosis and hemorrhages in the lungs.

Received for publication March 9, 1918.

[1] Virchows Arch., 1862, 25, p. 414.
[2] Ibid., 1880, 79, p. 168.
[3] Ztschr. f. Exp. Path. u. Ther., 1911, 9, p. 450.
[4] Ref. Handbook for Med. Sci., 154.
[5] Deut. Arch. f. klin. Med., 1915, 117, p. 602.

No other observers have corroborated the finding of coagulation of the blood in the vessels except Lassar, who found thrombi in the arteries of the viscera of chilled rabbits. Lang[6] did not find any coagulation from freezing localized areas, nor did Hochhaus[7] demonstrate thrombosis of the vessels in the kidney and liver to which he applied — 80° R. for 30 seconds.

Of the work done on establishing the rôle of cold as a factor in disease production and in the progress of disease already begun, Pasteur's experiments in 1878 on anthrax are the first to appear in the literature. These well known experiments were conducted as follows: Hens were inoculated with anthrax and half of them cooled in water, covering one third of their bodies, resulting in a fall of temperature from 42 to 38 C. All the chilled fowls developed the disease while the controls lived. Lock verified the results of Pasteur as did also Wagner,[8] who decreased the temperature by both antipyretics and the cold bath. Sawtschenko[9] obtained the same results with doves, and Dieudonne,[10] Petruchsky,[11] Nuttall,[12] and Metschnikoff[13] have shown that frogs are made susceptible to anthrax if they are warmed up and kept at 37 C.

Lode[14] in an extensive series of experiments found that guinea-pigs chilled in various ways were decidedly more susceptible to experimental infections with the pneumobacillus introduced either subcutaneously or by inhalation, with Staphylococcus aureus, cholera vibrio, B. anthracis and B. tuberculosis, than were controls similarly inoculated but not exposed to cold. Also, the mortality of animals already infected was shown to be increased greatly by exposure to cold. Lipari[15] and Rovighi[16] both found that chilled animals were much more susceptible to pneumococcus infections and the mortality was much higher than in control animals. Dürck[17] found that pneumonia developed without experimental infection in many of his animals which he had chilled in ice water, but never in his controls. Miller and Noble[18] found that chilled rabbits were decidedly more susceptible to B. bovisepticus (snuffles) than controls.

Ernst[19] injected frogs with B. ranicida and found that no infection resulted in the hot summer months, but in the cool spring or fall, or if the frogs were put in the ice-chest, they fell rapidly victims to the infection. Also he showed that if frogs were inoculated at a low temperature and a short time afterward heated up to summer temperature, no disease resulted. Filehne[20] found that after infecting rabbits' ears in the middle with erysipelas, the infection remained latent in the rabbits kept at 0 for 3 days, but severe and extensive erysipelas developed throughout the entire ear when the animal was transferred to room temperature, while in control rabbits the disease was much more moderate. He concludes that the bacteria had progressed to all parts of the ear by means of the lymph channels but that extensive growth was not allowed because of

[6] Abstract in Jour. Am. Med. Assn., 1915, 64.
[7] Virchows Arch., 1898, 154, p. 320.
[8] Centralbl. f. Bacteriol., 1891, 9, p. 322.
[9] Ibid., 1891-1899, pp. 473, 493, and 528.
[10] Arbeiten aus Kaiser. Gesundheitsamt., 1894, 9, p. 492.
[11] Beit. path. Anat., 1888, 3, p. 359.
[12] Ztschr. f. Hyg., 1888, 4, p. 353.
[13] Virchows Arch., 1884, 97, p. 502.
[14] Arch. f. Hyg., 1897, 28, p. 344.
[15] Deut. med. Wchnschr., 1890, 16, p. 44.
[16] Virchow and Hirsch Jahres. gesamt. Med., 1892, 1.
[17] Deut. Arch. f. klin. Med., 1897, 58, p. 368.
[18] Jour. Exper. Med., 1916, 24, p. 223.
[19] Beitr. path. Anat., 1890, 8, p. 213.
[20] Jour. Physiol., 1894-95, 17, p. 315.

anemia from the cold. Ciuca[21] and Vincent[22] demonstrated that tetanus could be produced in white mice inoculated with tetanus spores, by immersing for a considerable time in a freezing mixture. With rabbits, exposure of the entire animal was not sufficient to induce tetanus in animals injected with spores, but exposure of an injected extremity almost invariably resulted in the development of the disease. Vincent explains this phenomenon on the ground that the temperature of the isolated extremity is easily reduced to a temperature at which the phagocytic activity is destroyed, whereas it is impossible to decrease the temperature of the whole body to such a degree.

Ross and Williams[23] demonstrated that rats inoculated with trypanosomes (Evansi, Lewisi and Brucei) resisted the infection decidedly more than controls if they were kept in a cool chamber (20-36 F.). Ross and Thompson[24] showed the same thing for guinea-pigs inoculated with T. gambienzi.

Only two observers have failed to find a decreased resistance to bacterial infections when the host is exposed to cold. Pawlowsky[25] found that intense chilling—17 degrees for 15-30 minutes, failed to cause any decreased resistance to staphylococcus infections in rabbits, although many of his rabbits died from the cold directly. The other observer is Chodounsky,[26] who contests strenuously the belief that cold increases the susceptibility of laboratory animals or man to either artificial or autoinfection, especially referring to diseases of the respiratory tract. He cites his experiments on animals to the effect that pneumonia is not produced any easier in chilled than in control animals; he also presents results of experiments on himself to prove his point. He was 57 years old, subject to catarrh and frequent attacks of bronchitis, but nevertheless exposed himself after a hot bath, without drying, for periods of 20 minutes to an hour in draughts of cold air of 3-12 C. No pneumonia or serious results occurred.

Concerning the effect of cold on the cellular content of the blood, Rovighi, Winternitz,[27] and Schnützen[28] found that in both man and laboratory animals chilling causes a peripheral hyperleukocytosis, especially polymorphonuclear, which lasts for several hours, and Winternitz showed that there is considerable increase of the red cells (and increased hemoglobin index) in the peripheral blood. Rovighi and Secchi[29] showed that excessive cooling resulted in a decrease of the leukocytes, but an increase occurred when the heart action and respiration picked up again.

The conclusion to be drawn from the work on the effect of cold on phagocytosis, is that the leukocytes in chilled animals are less active than those of normal animals; and if leukocytes from normal animals are cooled in vitro their phagocytic activity is decreased. To this effect are the following experiments: Wagner demonstrated that the phagocytic activity of the leukocytes in hens injected with anthrax was reduced to a minimum in hens which had been cooled in a cold bath until the body temperature was reduced 2-3 C. Sanarelli[30] found that the leukocytes of chilled guinea-pigs were less phagocytic to Sp. metchnikovii than those of controls after the animals were chilled, while Lode, studying the

[21] Bull. de l'Acad. de méd., Feb. 17, 1908.
[22] Ibid., 1908.
[23] Ann. Trop. Med., 1910, 4,p. 225.
[24] Ibid., p. 510.
[25] Ztschr. Hyg. u. Infektionskr., 1900, 33, p. 289.
[26] Erkaltung und Erkaltungskrankheiten. Wien, 1907.
[27] Centralbl. f. klin. Med., 1893, 9, p. 1017.
[28] Wien. klin. Wchnschr., 1907, 20, p. 1625.
[29] Münch. med. Wchnschr., 1914, 61, p. 1721.
[30] Ann. de l'Inst. Past., 1893, 7, p. 225.

bacterial content of the leukocytes around the point of injection in the experiments cited, found that the leukocytes of control animals did not contain any more bacteria than the leukocytes in chilled animals. Dean[31] found that the action of opsonin on staphylococci was nearly 10 times as great at 37 as at 6 C., and Rolly and Meltzer[32] found that the phagocytic power of human leukocytes for typical bacilli increased gradually from 6 to 37.5 C., but quite rapidly from 37.5 to 40 C. Trommsdorff[33] found that in guinea-pigs which he injected intraperitoneally with hemolyzed hen corpuscles there was decreased migration of leukocytes into the cavity and that there was also a decreased phagocytosis of the corpuscles in the cooled animals. Ledingham[34] showed that when leukocytes and staphylococci were mixed in the presence of serum, the number of cocci ingested varied as the temperature, from 0 to 40 C., due to the fact that there was less action on the cocci by the opsonins in the serum at low temperatures. After a long sensitization at 18 or 7 C., the action of the leukocytes was quite independent of the temperature within certain limits as 7-18, 37, 40 C.

Practically all of the work on antibody production as influenced by cold has been done since 1906. One of the first series of experiments was that of Graziani,[35] who immunized rabbits with the filtrate from an 8-day broth culture of B. typhosus; three injections of increasing nonlethal doses were given under the skin of the back at intervals of two and three days. Briefly, he found that the agglutinin titer of the serum of animals kept constantly at 2-4 C. was nearly twice that of animals kept at room temperature as controls, the serum being drawn the same number of days after the last injection. In another series of experiments he kept half of a series of rabbits at 32 C. constantly during the experiment, while the other half were kept at 32 C. except for being chilled morning and evening during the period of immunization in water at 20 C. for 30 minutes, after which they were dried and put back at 32 C. The results from this series are similar to those in the first set of experiments, namely, the chilled rabbits showed on the average twice the agglutinin titer of the control animals. Graziani's method of determining the agglutinin titer was a microscopic one, the highest titer being the dilution in which there were no longer distinct clumps of 10-15 bacteria in the midst of free, slightly moving bacilli after 30 minutes.

Fukuhara,[36] in a rather incomplete experiment with 3 rabbits immunized to B. typhosus 31 days previously, found that the agglutinin and bacteriolysin content of the serum was increased in one animal cooled by dipping in cold water, this slight increase persisting for several days. The same result was obtained with a second animal kept at 10 C. for 3 hours, while keeping a third animal at 2 C. for 3 hours caused a higher agglutinin and bacteriolysin titer directly after the chilling, but the animal died on the following day and serum drawn while the animal was dying showed a decreased bacteriolysin titer while the agglutinin titer remained increased.

Trommsdorff, on the contrary, studying the agglutinin and bacteriolysin content of serum 1-8 days after the first injection of B. typhosus in guinea-pigs, found that strong cooling caused a considerable decrease in the agglutinin production. In these experiments the animals were immunized by injections of 1/10 lethal doses of typhoid bacilli, twice in the morning and twice in the afternoon on 2 successive days, and the tests for agglutination were made macro-

[31] Proc. Roy. Soc., 1905, 76, p. 706.
[32] Deut. Arch. f. klin. Med., 1908, 94, p. 335.
[33] Arch. f. Hyg., 1906, 59, p. 1-86.
[34] Centralbl. f. Bacteriol., I, O., 1908, 42, p. 632.
[35] Ibid., 1906, 42, p. 633.
[36] Arch. f. Hyg., 1908, 65, p. 275.

scopically. Trommsdorff showed also that guinea-pigs immunized to ox red blood cells (2 injections intraperitoneally of 5 c c washed red blood cells) showed a decreased hemolysin production after cooling during the period of immunization. In animals injected after cooling there was a decreased hemolysin production up to the 4th day after cooling. If the first injection was delayed until 5 or 6 days after the cooling there was no effect on the hemolysin production, showing that the effect of the cold had been overcome in that number of days. On a large number of animals Trommsdorff found that there was no constant effect produced on the alexin content of the blood by cooling the animals, as determined by hemolytic experiments. However, if the alexin content was decreased markedly by injection of large amounts of ox red blood cells the regeneration of alexin was very much slower in the cooled animals. He found also that there was a decreased extracellular lysis of partially hemolyzed hen's corpuscles injected into the peritoneal cavity of cooled animals.

Finally, Lissauer[37] found that chilling rabbits, which he had previously immunized with sheep corpuscles, in water at 10 C. to the extent that the body temperature reached 30-35.2 C., caused a marked reduction (about 4 times) in the hemolytic activity of the serum drawn immediately after cooling, compared with the activity of the serum drawn just before chilling. This phenomenon persisted 2-3 hours after the cooling, although tests were not made at later periods.

I. EFFECT OF CHILLING ON HEMOLYSIN PRODUCTION FOR SHEEP CORPUSCLES

Method of Experiment.—In this series 8 rabbits were used, 4 in the chilled group and 4 as controls. Rabbits of similar weight were put opposite one another in the chilled and control groups. All 8 rabbits were immunized with washed sheep corpuscles injected intravenously in doses as follows of 50% suspension at intervals of 2 days: 1 c c, 2 c c, 3 c c, 5 c c. The rabbits in the chilled group were immersed up to their necks in ice water, of which the temperature ranged from 0-8 C., for periods of 7-10 minutes twice a day during the period of immunization. On the days of injection the rabbits were chilled for this period 20 minutes after the injection, this delay being made to allow the immediate shock of the injection to pass off before the chilling was begun, and once again some time during the day after a lapse of 6-8 hours. On the days between the injections the animals were chilled twice, once in the morning and once in the evening. Of the original 8 rabbits, 3 died, one control dying acutely from the third injection, and two chilled rabbits, one dying from snuffles contracted 3 days after chilling began, and the other dying from the effect of cold directly. The first two of these rabbits were replaced by other rabbits treated as outlined. On an average the rectal temperature dropped 8 to 9 C., in one case 12 degrees and in another case 13 degrees, and did not reach normal again for 3 or 4 hours.

Blood was drawn from the ears on the days given in Table 1. In testing the hemolytic power of the serums after inactivation at 58 C. for ½ hour, each tube contained 3 c c, 1 c c being serums diluted with normal salt solution, 1 c c being 2½% suspension of sheep corpuscles and 1 c c being 1:20 guinea-pig serum, diluted with salt solution if necessary to make 1 c c. Before using the complement it was titrated against a known amboceptor so that sufficient but not an excess would be used. All tubes were incubated for 1 hour at 37 C. The figures give the highest dilution at which complete hemolysis occurred (Table 1).

[37] Arch. f. Hyg., 1907, 63, p. 332.

TABLE 1

HEMOLYTIC TITER OF SERUM OF DIFFERENT RABBITS

Days After First Injection	Days After Last Injection	Control Rabbits				Chilled Rabbits			
		1	2	5	7	2	4	6	8
6	...	132	133	133	80	133	133	133	133
9	3	800	800	800	800	800	800	1,333	1,000
12	6	2,000	2,000	4,000	2,000	4,000	2,000	3,000	3,000
16	10	2,000	1,333	3,000	2,000	3,000	1,333	2,000	2,000
20	14	2,000	1,333	2,000	1,000	2,000	1,000	1,000	1,000
23	17*	1,333	1,000	2,000	1,000	1,333	1,000	1,000	1,000
27	21*	400	200	1,333	500	1,000	200	dead	470
35	29*	200	150	1,000	300	500	170	dead	250

On the days marked with the asterisk the chilled rabbits were subjected to chilling 10 minutes at a time, blood being drawn immediately before chilling and immediately after (as in Lissauer's experiments) and the hemolytic titer of the serum determined. The results in Table 2 show that there was practically no difference between the 2 sets of serums.

TABLE 2

HEMOLYTIC TITER DETERMINED BEFORE AND AFTER CHILLING

Rabbit	Day After Last Injection	Titer Before Chilling	Titer After Chilling
2	17	1 : 1,333	1 : 1,333
	21	1 : 1,000	1 : 1,000
	29	500	667
4	17	1,000	1,000
	21	200	170
	29	170	170
6	17	1,000	1,000
	Dead	.....	.....
	Dead	.....	.....
8	17	1,000	1,000
	21	470	470
	29	250	250

From the data in Table 1, which gives the hemolytic titer of the serum from 8 rabbits for a period of 29 days both during and after the period of immunization, it is quite obvious that the chilling as conducted had practically no effect on the hemolysin production in Rabbits 2, 4, 6, and 8. The chilled rabbits ran as parallel to the control animals as possible, there being more individual variation between rabbits in either group than there was difference between the control and chilled groups. Trommsdorff's experience with guinea-pigs is contrary to this result, but his experiments were conducted in a different manner on a different species of animal and consequently there can be little more said in regard to the discrepancy.

Table 2 shows that the hemolysin content of the serum is not modified by chilling a rabbit in water for 10 minutes sufficiently to decrease the rectal temperature 8°–10° C. Lissauer's results, however, are entirely contrary to this result and I cannot explain or account for the discrepancies noted. If it was really the influence of cold which caused the antibody to decrease to one-fourth its concentration in the serum on account of chilling in 10° water in Lissauer's work, then it is hard to see why, if Lissauer's report is trustworthy, the rabbits in Table 2, chilled more vigorously than Lissauer's, did not show similar changes. It may be said that in my experiments the serum obtained immediately before and after chilling was titrated at the same time, the same corpuscle suspension, complement and salt solution being used, and the tubes were incubated the same length of time to the minute. Furthermore, it is hard to see how any such influence as cold could cause the removal of three-fourths of the hemolysin from the circulating blood.

II. EFFECT OF CHILLING ON THE AGGLUTININ PRODUCTION FOR BACILLUS TYPHOSUS

In this experiment 10 rabbits were used, 5 controls and 5 chilled animals, rabbits of similar weight being placed opposite one another in the 2 groups. The typhoid bacilli to be used in the immunization were grown on plain agar slants for 24 hours, the growth from each tube being washed off in 10 c c normal salt solution, and killed in a water bath at 60 C., for one-half hour. The following doses were given on the first 5 days: 0.25 c c, 0.3 c c, 0.3 c c, 0.35 c c, and 0.5 c c; 0.7 c c was given on the 7th day, 1.0 c c on the 8th, 1.3 c c on the 9th, 1.5 c c on the 10th, and 1.1 c c on the 13th. The animals of the chilled group were chilled as follows: 5 minutes on the 1st day, 6 on the 2nd, 7 on the 3rd, 7 on the 4th, 10 on the 5th, 10 on two occasions on the 6th, 10th and 7th, 12 on two occasions on the 8th, 12 on the 9th, 13 and 10 on the 10th, 12 on the 11th, 12 on the 12th, and 13 on the 13th. The method of chilling was the same as in the hemolytic experiment. The combination of cold and typhoid bacilli proved too much for 3 of the chilled rabbits, No. 4 dying on the 13th day, No. 8 on the 15th, and No. 10 on the 14th day. All the controls retained their weight and were in good health during the experiment except No. 5, which died from an accident on the 19th day.

In testing the agglutinin titer the macroscopic test tube method was used. The organisms to be agglutinated were grown on a large flat surface on plain agar in a large bottle for 24 hours, and then washed off in sufficient salt solution to make the desired turbidity. Before using they were killed by heating at 60 C. for one-half hour in the water bath. In making the tests each tube contained 1 c c of serum diluted with NaCl solution as necessary and 1 c c of typhoid suspension. All serums drawn on the same day were tested with the same typhoid suspension. All tubes were incubated 1 hour at 37 C., and the highest dilution in which agglutination was macroscopically visible was taken as the titer of the serum. Table 3 indicates the results.

TABLE 3

AGGLUTININ TITER ON DIFFERENT DAYS

Days After First Injection	Days After Last Injection	Control Rabbits 1	3	5	7	9	Chilled Rabbits 2	4	6	8	10
8	..	3,200	1,200	3,000	3,000	1,600	2,000	2,000	4,000	4,000	2,000
12	..	4,000	3,000	6,000	4,000	5,000	4,000	Dead	6,000	8,000	8,000
15	3	5,000	5,000	6,000	4,000	6,000	8,000	Dead	6,000	8,000	Dead
20	8	4,000	6,000	Dead	5,000	6,000	4,000	Dead	8,000	Dead	Dead
27	15	2,000	2,000	Dead	1,200	2,667	1,200	Dead	2,666	Dead	Dead

On the 15th day after the last injection the 6 rabbits alive at that time were chilled 10 minutes, blood being drawn immediately before chilling, 20 minutes after chilling, and 24 hours after chilling. Titration of the serums obtained gave the results shown in Table 4.

TABLE 4

AGGLUTININ TITER RECORDED AT DIFFERENT INTERVALS AFTER CHILLING

	1	2	3	4	7	9
Before chilling....................	2,000	1,200	2,000	2,666	1,200	2,666
20 minutes after chilling..........	2,000	1,600	2,666	4,000	1,600	4,000
24 hours after chilling............	2,000	1,600	2,666	4,000	1,600	4,000

From the data in Table 3 it is seen that chilling to the extent described causes a moderately increased agglutinin production for B. typhosus, the average titer in the chilled animals being higher than that of the control series. This was more obvious before the 3 chilled rabbits died, as the comparison of the titers of the first 3 sets of serums shows. Although the agglutinin production was increased the chilled rabbits were more susceptible to the typhoid injections, since 3 of 5 died and the remaining 2 lost more weight and fared much less favorably than the controls. This experiment corroborates Graziani rather than Trommsdorff, whose work on guinea-pigs gave just the opposite conclusions.

The data in Table 4 show that chilling rabbits immunized 15 days previously causes an increased agglutinin content in the serum (except in Rabbit 1), this increase persisting for 24 hours. This work corroborates Fukuhara's results.

ANATOMIC CHANGES PRODUCED IN THE LUNGS BY CHILLING

Aufrecht reported some experiments which seem not to have been repeated, and as a side issue to the chief topic of this paper, the following experiments were made to see whether Aufrecht's observations could be duplicated. Briefly, he reported experiments on 3 animals with no controls which he chilled by immersing in ice water for 1, 3 and 10 minutes, respectively, on each of 3 days. He found that chilling caused coagulation of fibrin in the blood stream in the

part chilled and in the lungs, the coagulation being ascribed by him to the injury to the white corpuscles by the cold. He states also that the coagulation in the lungs gave rise to hemorrhages, especially subpleural, and in the interstitial tissue. Migration of leukocytes was noticed into the adventitia of the vessels and also into the so-called thrombi. From the description the thrombi he observed might well be postmortem clots and artefacts produced by precipitation in the fixing fluid, and the special cell, "neutrophil lymphocyte," answers well the description of a polymorphonuclear leukocyte.

TABLE 5

RESULTS OF EXPERIMENTS TO DETERMINE WHETHER HEMORRHAGES AND BLOOD COAGULATION ARE DUE TO CHILLING

Rabbit	Time of Chilling	Method of Killing	Manner of Death	Macroscopic Changes	Microscopic Changes
1	1 minute on 3 successive days	Blow on neck	Severe spasms, 15 seconds	All showed numerous fresh red hemorrhages especially along margins of lungs, slightly more marked in rabbit No. 3. Hemorrhagic area varied from 1 to 8 mm. in diameter and extended into lung for 1 to 5 mm.	Fresh blood in alveoli in all sections from hemorrhagic areas. No edema. No coagulation except one small thrombus 2 mm. in diameter in No. 5. No cellular changes. No other changes
2	3 minutes on 3 successive days	Blow on neck	Severe spasms, 10 seconds		
3	10 minutes on 3 successive days	Blow on neck	Severe spasms, 20 seconds		
4	No chilling	Blow on neck	Severe spasms, 10 seconds		
5	No chilling	Blow on neck	Severe spasms, 15 seconds		
6	No chilling	Injection of ½ gm. KCN intravenous	Extremely severe spasms	Lungs riddled with many fresh red hemorrhages 2-10 mm. in diameter; half of lung tissue hemorrhagic	
7	No chilling	Intravenous injection of chloral (1 gm.)	Quiet death; no spasms or rigor	Several small fresh bright red hemorrhages (2-5 mm.) in upper lobes	
8	10 minutes on 3 successive days	Chloralized completely per rectum; blow on neck	Severe spasms	Several small areas of fresh, bright red hemorrhages in upper lobes; more than in No. 7	
9	10 minutes on 3 successive days	Intravenous chloral injection (1.3)	Quiet death; no spasms or rigor	Only few small areas of fresh hemorrhages; less than No. 7	
10	10 minutes on 3 successive days	Intravenous injection of .66 gm. chloral	Chest opened before death; heart cut open while still beating	One small area of fresh hemorrhages (2 mm.) in each of upper lobes	No changes except small area of fresh hemorrhage noted grossly
11	Same as No. 10	Same as No. 10	Same as No. 10	One small area (2 mm.) only in right upper lobe	No changes except small area of fresh hemorrhage noted grossly
12	Same as No. 10	Same as No. 10	Animal died before chest was opened. No spasms or rigor	Several small areas of fresh hemorrhages as in Nos. 7 and 9	No changes except small area of fresh hemorrhage noted grossly

In my experiments the first 3 rabbits (1, 2, and 3) were treated as Aufrecht's rabbits (Table 5). Rabbits 6 to 9 were narcotized, before chilling, by means of chloral injection per rectum (0.7 gm.). Rabbits 10, 11, 12 were not narcotized before chilling and were treated as follows with the results shown in Table 5.

The first conclusion to be drawn is that the hemorrhages in the lungs were in all cases *fresh* hemorrhages and apparently occurred at the time of death. Furthermore, the hemorrhages occurring in the lungs of rabbits killed by means of a blow on the neck were not due to the chilling, for the controls in all cases showed as much hemorrhage as the chilled animals. Hence, the results indicate that the hemorrhages described by Aufrecht were agonal. In all cases, the amount of hemorrhage in the lung varied with the amount of spasm and rigor as the animal died. The possibility that some of the hemorrhages noticed, especially the more obscure, deeper ones, might have resulted from the struggles in the bath, led to the narcotization of Rabbits 6-9, inclusive, before chilling. However, since the findings in these rabbits are similar to those in the unnarcotized Rabbit 12 killed in a similar manner and to the findings in narcotized rabbits not subjected to cooling (No. 7), and since rabbits not narcotized and which struggled when chilled showed practically no hemorrhages when killed in a different way (Nos. 10 and 11), it is obvious that no hemorrhages resulted from the muscular efforts of the rabbits when being chilled.

Finally, the conclusion is reached that cold did not cause coagulation in the vessels of the lungs, the only thrombus found in the whole series being noticed in a control animal. Numerous structures corresponding to those described by Aufrecht as thrombi were noticed in all the lungs, but they were merely postmortem clots and artefacts produced by fixation.

SUMMARY

Chilling rabbits twice a day during the period of immunization for periods of 7-10 minutes in water at 8° C. did not cause any change in the hemolysin production for sheep corpuscles, and a single chilling 17-29 days after the period of immunization failed to change the hemolysin content of the serum.

Chilling rabbits thoroughly during the period of immunization caused a moderate increase in agglutinin production for B. typhosus. Chilling once 15 days after the period of immunization caused an increase in the agglutinin content of the serum, which increase persisted for 24 hours.

Cold baths in ice water at 0° C. for 1–10 minutes failed to cause hemorrhages or thrombosis in the lungs of rabbits.

"HORMONE" MEDIUM

A SIMPLE MEDIUM EMPLOYABLE AS A SUBSTITUTE FOR SERUM MEDIUM

F. M. Huntoon, Contract Surgeon, U. S. Army

From Department of Bacteriology, Cornell University Medical School, New York City, and Base Hospital Laboratory, Camp Merritt, N. J.

The investigations of many workers have long been directed toward the production of mediums that would answer the growth requirements of delicate organisms without the necessity of employing serous fluids — a complicated technic. The list of such mediums is long and many have answered the necessary requirements in part, while the work of English observers[1] in recent years, has pointed out the factors which are concerned in the reproduction of bacteria on artificial medium and in particular the part played by the so-called vitamins or hormones in establishing and conserving bacterial growth and the importance of the presence of suitable amino-acids.

Such mediums as they prepared are rather complicated and the ingredients are not always to be obtained, so that it seemed possible and necessary to attempt the production of mediums based on these considerations, but with such a simplified technic that it would be readily available under field conditions. Accordingly, various mediums were prepared, keeping the following factors in mind:

1. To extract the growth factors or "hormones" by bringing colloidal solutions, in this case melted agar or gelatin, into contact with the meat and blood.

2. To preserve these factors by limiting the amount of heating as much as possible, and most important, not to filter these solutions at any time through cloth, cotton or filter paper, since the "hormones" are readily adsorbed by vegetable fiber — one passage through filter paper of the finished product removing half of its initial growth value.

3. To furnish sufficient amino-acids by the use of a suitable peptone and the addition of egg-yolk.

4. To keep the hydrogen ion content in the proper zone, although this factor when the other conditions are suitable is not as important as has been assumed.

5. To so simplify the technic that the mediums could be produced under the most unfavorable conditions.

Received for publication June 7, 1918.

[1] Lloyd: Jour. Path. and Bact., 1916, Vol. 21, Pt. I, p. 113; Cole and Lloyd: Ibid., 1917, Vol. 21, Pt. 2, p. 267.

Many mediums were prepared and various organs employed as a basis, and it was evident from the first that the beef usually employed and especially beef heart furnished sufficient "hormones" for the purpose, and that the question of getting suitable medium was merely that of a suitable technic.

The following method was finally developed as being most generally satisfactory:

"HORMONE" AGAR

	Gm.
Chopped beef heart or steak (must be comparatively fresh)	500
Water	1,000
Peptone (Bacto peptone gives the best results)	10
Agar (Bacto or thread agar that has been soaked)	16
Salt	5
Whole egg	1

All of these ingredients are placed in an ordinary enamel ware vessel, preferably a large coffee pot, and heated over an open flame with constant stirring until the red color of the meat infusion changes to brown, at a temperature about 68 C. Care should be taken not to run the temperature much above this point as the medium then begins to clot, which is undesirable at this time.

The medium is now titrated by the addition of normal sodium hydrate until it is slightly alkaline to litmus paper, and then 1 c c per liter is added in addition.

The vessel is covered and placed in the Arnold sterilizer or in a water bath at a temperature of 100 C. for 1 hour, removed, and the firm clot which has formed separated from the sides with a rod and the vessel returned to the sterilizer or water bath at 100 C. for 1½ hours.

It is now removed and allowed to stand at room temperature for about 10 minutes in a slightly inclined position; during this time the fluid portion separates and may be removed by pipetting or, in the case of the coffee pot, by simply pouring it off carefully. If it is poured through a fine wire sieve many small particles of meat clot may be caught.

The product is allowed to stand in tall cylinders for 15 to 20 minutes, until the fat present has risen to the surface where it can be removed. The medium is now tubed and sterilized by the intermittent method. Autoclaving is to be avoided.

If the medium, although usually clear enough for practical purposes, seems too turbid, further clearing may take place by filtration through glass wool, asbestos wool, sedimentation or centrifugation.

The product prepared according to the foregoing directions is available for all the usual laboratory uses and in addition has a growth value 10 times as great as standard agar and is at least as good as the average grade serum agar.

"HORMONE" SEMISOLID AGAR

Prepared as directed with the substitution of 5 gm. for 16 gm. of agar, this medium is tubed in about 10 c c amounts and employed for stab cultivations, and is an extremely useful medium both for

anaerobes and aerobes, and is especially suitable for the preservation of stock cultures. Experiments with the group of gram-negative cocci shows the meningococcus when sealed up in this medium and kept in the incubator to live for 3 months without transfer and the gonococcus for 2 months.

HORMONE GELATIN BROTH

Since an essential feature of the preparation of these mediums is the contact of colloidal solutions with the meat infusion the necessity arises of the addition of a small percentage of gelatin in the fluid medium. This is done by the addition of 10 gm. of gelatin as a substitute for the agar, otherwise the method is unchanged.

Such a medium is liquid at room and incubator temperatures and the presence of the gelatin in no way interferes with the usefulness of the product. It was feared that the gelatin might interfere with agglutination and precipitin tests for determination of type of the pneumococcus, but such proved not to be the case, the reactions being as sharp and prompt as in the medium usually employed for this purpose. Growth of practically all organisms tried is more rapid, more marked, and their peculiar characteristics are exaggerated, rendering tentative diagnoses easier to make. Capsule production by the pneumococcus is usually evident when inoculated from body fluids or organs rendering the use of serum medium unnecessary for this purpose.

MODIFICATIONS FOR SPECIAL PURPOSES

It was found that the "hormone" agar was as suitable for meningococcus contact cultivations as the mediums recommended for this purpose. Later, at the suggestion of Miss Anna Williams of the New York Health Department, the addition of small amounts (about 1 c c to the 100 c c) of defibrinated blood or better of laked blood (just enough to give a pinkish tinge to the poured plate) rendered the medium on actual test superior to glucose-ascitic agar as far as number of initial colonies established and size of colony are concerned, and has superseded other mediums for this type of work.

I have had the opportunity of testing the values of this modification in actual field work on many cases and in direct comparison to the standard mediums and have found, when experienced workers in this field are offered the opportunity of direct comparison of results, not to speak of the convenience the simplicity of preparation gives, that the decision is in favor of the newer medium. For general work

it may be employed successfully wherever ascitic agar would be indicated.

A modification of the "hormone" gelatin broth is by the addition of 0.15% dextrose and the addition of enough laked blood to give it a slight pink tint. This medium has remarkable growth value, the streptococcus showing a very distinct and marked growth in 4 hours, and in 18 hours a thick heavy mass of tangled chains at the bottom of the tube extending up about one-quarter of the height of the column of liquid, and when shaken up being as densely turbid as the growth usually seen on a 24-hour colon-broth culture.

When tried with spinal fluid from meningitis cases in which no organisms could be found in smears, distinct growth occurred in 9 hours, rendering a positive diagnosis possible. A peculiar feature of this growth is the fact that the organisms multiplied within the leukocytes transferred.

This medium is favorable for the growth of the pneumococcus, which gives a marked turbidity. Smears from the growth show well marked capsules.

Another modification, but not of such general utility, is the preparation of a substitute for Dorset's egg medium.

In this medium, which is prepared in a similar manner as the hormone agar, 1% aminoids are substituted for peptone and 5% glycerol is added to the finished product; the tubercle bacillus grows in 10 days almost as vigorously as on Dorset's egg medium.

Many other modifications and uses of this type of medium will doubtless suggest themselves and the fact that here is presented a simple utilization of several well known and demonstrated principles of bacterial growth, leads to the hope that other workers in this field will further improve on the methods.

SUMMARY

A basis for the production of laboratory mediums is given in which the points to be remembered are the extraction of the growth factors by colloidal solutions and avoidance of filtering by passage through any cloth, filter paper or cotton.

The advantages of medium so prepared are: Simplicity of preparation technic; great increase in growth efficiency; prolonged life of stock cultures, and convenience in the preparation of bacterial antigens, there being no serum element to be taken into consideration.

THE INFLUENCE OF INCUBATION ON THE WASSERMANN REACTION

E. H. Ruediger

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

Smith and MacNeal[1] demonstrated that widely different results may be obtained in complement-fixation for syphilis by the use of different incubation methods. First incubation conducted in the refrigerator at a low temperature gave many more positive results than did first incubation conducted at a temperature of 37 C. In a later communication[2] the same authors showed that cholesterinized antigen, plain alcoholic extract, and the acetone insoluble antigen bind much more complement at low temperature than at a temperature of 37 C. In the refrigerator, plain alcoholic extract bound more complement than did cholesterinized antigen in the incubator.

In this paper I shall present the results I have obtained with the Wassermann reaction under different incubation conditions. Only one antigen was employed, and the only difference there was, was in incubation time and method.

The results are presented under 5 different heads. Under the first head are given results obtained by comparing incubation in the incubator at a temperature of 37 C. with incubation in the open water-bath at a temperature of 37 C. The second deals with results obtained in the incubator at 37 C. and in the refrigerator at 10 C. Here each serum was tested 10 times, first incubation in incubator 0.25 hour, 0.5 hour, 1 hour, 2 hours and 4 hours, and these were duplicated in the refrigerator. In the third group each serum was tested 4 times; first incubation was carried on in the incubator for 1 and 4 hours, and in the refrigerator for 1 and 4 hours. In the fourth group the incubation time only was varied. First incubation was carried on in the refrigerator for 5 and 10 hours. In the fifth group first incubation at a temperature of 21 C. for 5 hours was compared with first incubation at a temperature of 10 C. for 5 hours. All human serums used in these tests were glycerolated, and in Tests 1 and 2 each tube received 0.1 c c of serum-glycerol mixture while in Tests 3, 4, and 5 each tube received 0.25 c c of serum-glycerol mixture and the total quantity in each tube was brought up to 1.25 c c.

Test 1

Serums 1 to 20, inclusive, were tested 4 times. First incubation was in the incubator at 37 C. for 1 and 2 hours, and in the water-bath at 37 C. for 1 and 2 hours. After having added the sensitized blood corpuscles the

Received for publication, March 12, 1918.

[1] Jour. Immunol., 1916, 2, p. 75.

[2] Jour. Infect. Dis., 1917, 21, p. 233.

TABLE 1

INCUBATION IN INCUBATOR COMPARED WITH INCUBATION IN WATER-BATH

Number	Incubation Method I = Incubator W = Water-bath	Incubation Time, Hours	Amboceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1′	2′	3′		
1	I	1	1.25	+	+	tr	+	+	tr	Negative,	—
	W	1	1.25	+	+	0	+	+	0	Negative,	—
	I	2	1.25	+	+	tr	+	+	tr	Negative,	—
	W	2	1.25	+	+	0	+	+	0	Negative,	—
2	I	1	1.25	+	+	tr	+	+	tr	Negative,	—
	W	1	1.25	+	+	0	+	+	0	Negative,	—
	I	2	1.25	+	+	tr	+	+	tr	Negative,	—
	W	2	1.25	+	+	0	+	+	0	Negative,	—
3	I	1	1.25	+	+	tr	+	+	tr	Negative,	—
	W	1	1.25	+	+	0	+	+	0	Negative,	—
	I	2	1.25	+	+	tr	+	+	tr	Negative,	—
	W	2	1.25	+	+	0	+	+	0	Negative,	—
4	I	1	1.25	+	+	±	+	+	±	Negative,	—
	W	1	1.25	+	+	tr	+	+	tr	Negative,	—
	I	2	1.25	+	+	±	+	+	±	Negative,	—
	W	2	1.25	+	+	tr	+	+	tr	Negative,	—
5	I	1	1.25	+	+	±	+	+	±	Negative,	—
	W	1	1.25	+	+	tr	+	+	tr	Negative,	—
	I	2	1.25	+	+	±	+	+	±	Negative,	—
	W	2	1.25	+	+	tr	+	+	tr	Negative,	—
6	I	1	1.25	+	+	±	+	+	±	Negative,	—
	W	1	1.25	+	+	tr	+	+	tr	Negative,	—
	I	2	1.25	+	+	±	+	+	±	Negative,	—
	W	2	1.25	+	+	tr	+	+	tr	Negative,	—
7	I	1	1.25	+	+	±	+	+	±	Negative,	—
	W	1	1.25	+	+	tr	+	+	tr	Negative,	—
	I	2	1.25	+	+	±	+	+	±	Negative,	—
	W	2	1.25	+	+	tr	+	+	tr	Negative,	—
8	I	1	1.25	+	+	±	+	+	±	Negative,	—
	W	1	1.25	+	+	±	+	+	±	Negative,	—
	I	2	1.25	+	+	±	+	+	±	Negative,	—
	W	2	1.25	+	+	tr	+	+	tr	Negative,	—
9	I	1	1.25	+	+	tr	+	+	tr	Negative,	—
	W	1	1.25	+	+	0	+	+	0	Negative,	—
	I	2	1.25	+	+	tr	+	+	tr	Negative,	—
	W	2	1.25	+	+	0	+	+	0	Negative,	—
10	I	1	1.25	+	+	tr	+	+	tr	Negative,	—
	W	1	1.25	+	+	0	+	+	0	Negative,	—
	I	2	1.25	+	+	tr	+	+	tr	Negative,	—
	W	2	1.25	+	+	0	+	+	0	Negative,	—
11	I	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	W	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	W	2	1.25	+	+	0	+	+	tr	Weakly positive,	+
12	I	1	1.25	+	±	0	+	+	±	Strongly positive,	+++
	W	1	1.25	+	+	0	+	+	±	Moderately positive,	++
	I	2	1.25	+	tr	0	+	+	±	Strongly positive,	++++
	W	2	1.25	+	+	0	+	+	±	Moderately positive,	++
13	I	1	1.25	+	+	0	+	+	±	Moderately positive,	++
	W	1	1.25	+	+	0	+	+	±	Moderately positive,	++
	I	2	1.25	+	+	0	+	+	±	Moderately positive,	++
	W	2	1.25	+	+	0	+	+	±	Moderately positive,	++

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

TABLE 1—*Continued*

INCUBATION IN INCUBATOR COMPARED WITH INCUBATION IN WATER-BATH

Number	Incubation Method I = Incubator W = Water-bath	Incubation Time, Hours	Amboceptor per Tube Unit	Readings: Antigen Tubes			Readings: Control Tubes			Results	
				1	2	3	1′	2′	3′		
14	I	1	1.25	+	+	0	+	+	tr	Weakly positive,	+
	W	1	1.25	+	+	0	+	+	tr	Weakly positive,	+
	I	2	1.25	+	+	0	+	+	±	Moderately positive,	++
	W	2	1.25	+	+	0	+	+	tr	Weakly positive,	+
15	I	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	W	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	2	1.25	+	+	0	+	+	±	Moderately positive,	++
	W	2	1.25	+	±	0	+	+	tr	Moderately positive,	++
16	I	1	1.25	+	0	0	+	+	tr	Strongly positive,	++++
	W	1	1.25	+	tr	0	+	+	tr	Strongly positive,	+++
	I	2	1.25	+	0	0	+	+	±	Strongly positive,	+++++
	W	2	1.25	+	tr	0	+	+	tr	Strongly positive,	+++
17	I	1	1.25	+	+	0	+	+	±	Moderately positive,	++
	W	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	2	1.25	+	±	0	+	+	±	Strongly positive,	+++
	W	2	1.25	+	±	0	+	+	tr	Moderately positive,	++
18	I	1	1.25	+	+	0	+	+	±	Moderately positive,	++
	W	1	1.25	+	+	0	+	+	tr	Weakly positive,	+
	I	2	1.25	+	+	0	+	+	±	Moderately positive,	++
	W	2	1.25	+	+	0	+	+	tr	Weakly positive,	+
19	I	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	W	1	1.25	+	+	0	+	+	tr	Weakly positive,	+
	I	2	1.25	+	+	0	+	+	tr	Moderately positive,	++
	W	2	1.25	+	+	0	+	+	tr	Weakly positive,	+
20	I	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	W	1	1.25	+	tr	0	+	+	0	Moderately positive,	++
	I	2	1.25	+	±	0	+	+	tr	Moderately positive,	++
	W	2	1.25	+	tr	0	+	+	0	Moderately positive,	++

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

tubes from the incubator were returned to the incubator, and the tubes from the water-bath were returned to the water-bath.

Table 1 shows the results obtained in the incubator and in the water-bath with Serums 1-20, inclusive. The first 10 serums were from nonsyphilitic persons, and they all gave negative results. Serums 11 to 20, inclusive, were from persons known to be syphilitic. During the first incubation, complement was bound better in the incubator than in the water bath; Serum 12 gave + + + and + + + + in the incubator, and + + and + + in the water-bath; Serums 14, 16, 17, 18, and 19 also gave stronger positive results in the incubator than in the water-bath. In no instance was the result obtained in the water-bath stronger positive than that obtained in the incubator.

TEST 2

In order to observe the rate at which complement is bound Serums 21-30, inclusive, were incubated in the incubator at a temperature of 37 C. and in the refrigerator at a temperature of 10 C. for 0.25 hour, 0.5 hour, 1 hour, 2 hours, and 4 hours. After the addition of sensitized blood corpuscles all tubes were placed in the incubator because hemolysis was better in the incubator than in the water-bath. All of these serums were obtained from syphilitic patients under treatment.

TABLE 2

INCUBATION IN INCUBATOR COMPARED WITH INCUBATION IN REFRIGERATOR

Number	Incubation Method I = Incubator R = Refrigerator	Incubation Time, Hours	Amboceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1′	2′	3′		
21	I	0.25	1.25	+	+	±	+	+	±	Negative,	—
	R	0.25	1.25	+	+	±	+	+	±	Negative,	—
	I	0.5	1.25	+	+	±	+	+	±	Negative,	—
	R	0.5	1.25	+	+	±	+	+	±	Negative,	—
	I	1	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	1	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	4	1.25	+	±	0	+	+	tr	Moderately positive,	++
	R	4	1.25	+	+	0	+	+	±	Moderately positive,	++
22	I	0.25	1.25	+	+	±	+	+	±	Negative,	—
	R	0.25	1.25	+	+	±	+	+	±	Negative,	—
	I	0.5	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	0.5	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	4	1.25	+	±	0	+	+	tr	Moderately positive,	++
	R	4	1.25	+	+	0	+	+	±	Moderately positive,	++
23	I	0.25	1.25	+	+	±	+	+	±	Negative,	—
	R	0.25	1.25	+	+	±	+	+	±	Negative,	—
	I	0.5	1.25	+	+	±	+	+	±	Negative,	—
	R	0.5	1.25	+	+	±	+	+	±	Negative,	—
	I	1	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	1	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	2	1.25	+	+	0	+	+	tr	Weakly positive,	+
	R	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	4	1.25	+	+	0	+	+	tr	Weakly positive,	+
	R	4	1.25	+	+	tr	+	+	±	Weakly positive,	+
24	I	0.25	1.25	+	+	±	+	+	±	Negative,	—
	R	0.25	1.25	+	+	±	+	+	±	Negative,	—
	I	0.5	1.25	+	+	±	+	+	±	Negative,	—
	R	0.5	1.25	+	+	±	+	+	±	Negative,	—
	I	1	1.25	+	+	tr?	+	+	tr	Faintly positive,	±
	R	1	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	2	1.25	+	±	0	+	+	0	Weakly positive,	+
	R	2	1.25	+	+	0	+	+	tr	Weakly positive,	+
	I	4	1.25	+	tr	0	+	+	0	Moderately positive,	++
	R	4	1.25	+	±	0	+	+	tr	Moderately positive,	++
25	I	0.25	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	0.25	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	0.5	1.25	+	+	0	+	+	±	Moderately positive,	++
	R	0.5	1.25	+	±	0	+	+	±	Strongly positive,	+++
	I	1	1.25	+	±	0	+	+	±	Strongly positive,	+++
	R	1	1.25	+	0	0	+	+	±	Strongly positive,	+++++
	I	2	1.25	+	tr	0	+	+	±	Strongly positive,	++++
	R	2	1.25	+	0	0	+	+	±	Strongly positive,	+++++
	I	4	1.25	+	0	0	+	+	tr	Strongly positive,	++++
	R	4	1.25	±	0	0	+	+	±	Strongly positive,	++++++
26	I	0.25	1.25	+	±	0	+	+	tr	Moderately positive,	++
	R	0.25	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	0.5	1.25	+	±	.0	+	+	tr	Moderately positive,	++
	R	0.5	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	R	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	2	1.25	+	tr	0	+	+	0	Moderately positive,	++
	R	2	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	4	1.25	+	0	0	+	+	0	Strongly positive,	+++
	R	4	1.25	+	tr	0	+	+	tr	Strongly positive,	+++

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

TABLE 2—*Continued*

INCUBATION IN INCUBATOR COMPARED WITH INCUBATION IN REFRIGERATOR

Number	Incubation Method I = Incubator R = Refrigerator	Incubation Time, Hours	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results	
27	I	0.25	1.25	+	+	0	+	+	tr	Weakly positive,	+
	R	0.25	1.25	+	+	0	+	+	tr	Weakly positive,	+
	I	0.5	1.25	+	±	0	+	+	tr	Moderately positive,	++
	R	0.5	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	R	1	1.25	+	±	0	+	+	tr	Moderately positive,	++
	I	2	1.25	+	tr	0	+	+	0	Moderately positive,	++
	R	2	1.25	+	tr	0	+	+	tr	Strongly positive,	+++
	I	4	1.25	+	0	0	+	+	0	Strongly positive,	+++
	R	4	1.25	+	tr	0	+	+	tr	Strongly positive,	+++
28	I	0.25	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	0.25	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	0.5	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	0.5	1.25	+	±	0	+	+	±	Strongly positive,	+++
	I	1	1.25	+	±	0	+	+	±	Strongly positive,	+++
	R	1	1.25	+	tr	0	+	+	±	Strongly positive,	++++
	I	2	1.25	+	±	0	+	+	±	Strongly positive,	+++
	R	2	1.25	+	tr	0	+	+	±	Strongly positive,	++++
	I	4	1.25	+	tr	0	+	+	tr	Strongly positive,	+++
	R	4	1.25	+	0	0	+	+	±	Strongly positive,	+++++
29	I	0.25	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	0.25	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	0.5	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	0.5	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	4	1.25	+	+	0	+	+	±	Moderately positive,	++
	R	4	1.25	+	+	0	+	+	±	Moderately positive,	++
30	I	0.25	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	0.25	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	0.5	1.25	+	+	±?	+	+	±	Faintly positive,	±
	R	0.5	1.25	+	+	±?	+	+	±	Faintly positive,	±
	I	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	1	1.25	+	+	tr	+	+	±	Weakly positive,	+
	I	2	1.25	+	+	tr	+	+	±	Weakly positive,	+
	R	2	1.25	+	+	0	+	+	±	Moderately positive,	++
	I	4	1.25	+	±	0	+	+	tr	Moderately positive,	++
	R	4	1.25	+	+	0	+	+	±	Moderately positive,	++

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

The results obtained with Serums 21-30, inclusive, are shown in Table 2. Binding of complement proceeded slowly and was a trifle faster in the refrigerator than in the incubator. Serum 28, after 0.25 hour incubation, gave a faintly positive result by both methods. After 0.5 hour incubation the incubator method gave a weakly positive (+) result and the refrigerator method gave a strongly positive (+ + +) result. With 4 hour incubation the incubator method gave a + + + positive result and the refrigerator method gave a + + + + + positive result. Serum 25 shows the same difference, and none of the other serums gave stronger positive results by the incubator method than by the refrigerator method.

TEST 3

With serums 31 to 50, inclusive, first incubation was done in the incubator and in the refrigerator for 1 and 4 hours. In these tests larger quantities of human serum were used than in Tests 1 and 2, hence the larger quantities of hemolytic amboceptor. After having added the sensitized blood corpuscles all tubes were placed in the incubator, the water-bath having been discarded. Serums 31 to 40, inclusive, came from nonsyphilitic persons and Serums 41 to 50, inclusive, came from known syphilitics.

TABLE 3

INCUBATION IN INCUBATOR COMPARED WITH INCUBATION IN REFRIGERATOR

Number	Incubation Method I = Incubator R = Refrigerator	Incubation Time, Hours	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results	
31	I	1	1.5	+	+	±	+	+	±	Negative,	—
	R	1	1.5	+	+	±	+	+	±	Negative,	—
	I	4	1.5	+	+	tr	+	+	tr	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative,	—
32	I	1	1.5	+	+	tr	+	+	tr	Negative,	—
	R	1	1.5	+	+	±	+	+	±	Negative,	—
	I	4	1.5	+	+	0	+	+	0	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative.	—
33	I	1	1.5	+	+	±	+	+	±	Negative,	—
	R	1	1.5	+	+	±	+	+	±	Negative,	—
	I	4	1.5	+	+	tr	+	+	tr	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative,	—
34	I	1	1.5	+	+	±	+	+	±	Negative,	—
	R	1	1.5	+	+	±	+	+	±	Negative,	—
	I	4	1.5	+	+	tr	+	+	tr	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative,	—
35	I	1	1.5	+	+	±	+	+	±	Negative,	—
	R	1	1.5	+	+	±	+	+	±	Negative,	—
	I	4	1.5	+	+	tr	+	+	tr	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative,	—
36	I	1	1.5	+	+	tr	+	+	tr	Negative,	—
	R	1	1.5	+	+	tr	+	+	tr	Negative,	—
	I	4	1.5	+	+	tr	+	+	tr	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative,	—
37	I	1	1.5	+	+	tr	+	+	tr	Negative,	—
	R	1	1.5	+	+	±	+	+	±	Negative,	—
	I	4	1.5	+	+	tr	+	+	tr	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative,	—
38	I	1	1.5	+	+	tr	+	+	tr	Negative,	—
	R	1	1.5	+	+	±	+	+	±	Negative,	—
	I	4	1.5	+	+	tr	+	+	tr	Negative,	—
	R	4	1.5	+	+	±	+	+	±	Negative,	—
39	I	1	1.5	+	+	tr	+	+	tr	Negative,	—
	R	1	1.5	+	+	tr	+	+	tr	Negative,	—
	I	4	1.5	+	+	0	+	+	0	Negative,	—
	R	4	1.5	+	+	tr	+	+	tr	Negative,	—
40	I	1	1.5	+	+	tr	+	+	tr	Negative,	—
	R	1	1.5	+	+	tr	+	+	tr	Negative,	—
	I	4	1.5	+	+	0	+	+	0	Negative,	—
	R	4	1.5	+	+	tr	+	+	tr	Negative,	—

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

TABLE 3—*Continued*

INCUBATION IN INCUBATOR COMPARED WITH INCUBATION IN REFRIGERATOR

Number	Incubation Method I = Incubator R = Refrigerator	Incubation Time, Hours	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results
41	I	1	1.5	+	±	0	+	+	tr	Moderately positive, ++
	R	1	1.5	+	0	0	+	+	tr	Strongly positive, ++++
	I	4	1.5	+	±	0	+	+	tr	Moderately positive, ++
	R	4	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++
42	I	1	1.5	+	+?	0	+	+	0	Faintly positive, ±
	R	1	1.5	+	±	0	+	+	0	Weakly positive, +
	I	4	1.5	+	tr	0	+	+	0	Moderately positive, ++
	R	4	1.5	+	0	0	+	+	0	Strongly positive, +++
43	I	1	1.5	+	+	tr?	+	+	tr	Faintly positive, ±
	R	1	1.5	+	+	tr	+	+	±	Weakly positive, +
	I	4	1.5	+	+	0	+	+	tr	Weakly positive, +
	R	4	1.5	+	tr	0	+	+	±	Strongly positive, ++++
44	I	1	1.5	+	+	±	+	+	±	Negative, —
	R	1	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++
	I	4	1.5	+	+	tr	+	+	tr	Negative, —
	R	4	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++
45	I	1	1.5	+	+	±	+	+	±	Negative, —
	R	1	1.5	+	tr	0	+	+	tr	Strongly positive, ++++
	I	4	1.5	+	+	tr	+	+	tr	Negative, —
	R	4	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++
46	I	1	1.5	+	+	tr	+	+	±	Weakly positive, +
	R	1	1.5	+	±	0	+	+	±	Strongly positive, +++
	I	4	1.5	+	+	0	+	+	tr	Weakly positive, +
	R	4	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++
47	I	1	1.5	+	+	±	+	+	±	Negative, —
	R	1	1.5	+	+	±	+	+	±	Negative, —
	I	4	1.5	+	+	tr	+	+	tr	Negative, —
	R	4	1.5	+	±	0	+	+	±	Strongly positive, +++
48	I	1	1.5	+	+	0	+	+	±	Moderately positive, ++
	R	1	1.5	+	±	0	+	+	±	Strongly positive, +++
	I	4	1.5	+	+	0	+	+	±	Moderately positive, ++
	R	4	1.5	+	tr	0	+	+	±	Strongly positive, ++++
49	I	1	1.5	+	+	0	+	+	±	Moderately positive, ++
	R	1	1.5	+	tr	0	+	+	±	Strongly positive, ++++
	I	4	1.5	+	±	0	+	+	±	Strongly positive, +++
	R	4	1.5	±	0	0	+	+	±	Strongly positive, ++++++
50	I	1	1.5	+	tr	0	+	+	±	Strongly positive, ++++
	R	1	1.5	±	0	0	+	+	±	Strongly positive, ++++++
	I	4	1.5	+	0	0	+	+	±	Strongly positive, +++++
	R	4	1.5	0	0	0	+	+	±	Strongly positive, +++++ +++++

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

In Table 3 are recorded the results obtained with Serums 31 to 50, inclusive. Serums 31 to 40, inclusive, gave negative results with all 4 methods. With Serums 41 to 50 the results varied greatly; such results as those obtained with Serums 44 and 45 may be very unusual. All of these serums gave much stronger positive results by the refrigerator method than by the incubator method, and with 4-hour first incubation much stronger positive results were obtained than with 1-hour first incubation.

TABLE 4

Five Hour Incubation in Refrigerator Compared with Ten Hour Incubation in Refrigerator

Number	Incubation Method R = Refrigerator	Incubation Time, Hours	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results	
51	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
52	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
53	R	5	1.5	+	+	tr	+	+	tr	Negative,	—
	R	10	1.5	+	+	tr	+	+	tr	Negative,	—
54	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
55	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
56	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
57	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
58	R	5	1.5	+	+	tr	+	+	tr	Negative,	—
	R	10	1.5	+	+	tr	+	+	tr	Negative,	—
59	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
60	R	5	1.5	+	+	±	+	+	±	Negative,	—
	R	10	1.5	+	+	±	+	+	±	Negative,	—
61	R	5	1.5	+	±	0	+	+	±	Strongly positive,	+++
	R	10	1.5	+	tr	0	+	+	±	Strongly positive,	++++
62	R	5	1.5	+	tr	0	+	+	±	Strongly positive,	++++
	R	10	1.5	+	0	0	+	+	±	Strongly positive,	+++++
63	R	5	1.5	+	tr	0	+	+	±	Strongly positive,	++++
	R	10	1.5	+	0	0	+	+	±	Strongly positive,	+++++
64	R	5	1.5	+	+	tr	+	+	±	Weakly positive,	+
	R	10	1.5	+	+	0	+	+	±	Moderately positive,	++
65	R	5	1.5	+	+	0	+	+	±	Moderately positive,	++
	R	10	1.5	+	tr	0	+	+	±	Strongly positive,	++++
66	R	5	1.5	+	tr	0	+	+	±	Strongly positive,	++++
	R	10	1.5	tr	0	0	+	+	±	Strongly positive,	++++
											++++
67	R	5	1.5	+	+	±?	+	+	±	Faintly positive,	±
	R	10	1.5	+	+	tr	+	+	±	Weakly positive,	+
68	R	5	1.5	+	+	0	+	+	±	Moderately positive,	++
	R	10	1.5	+	±	0	+	+	±	Strongly positive,	+++
69	R	5	1.5	+	+	0	+	+	±	Moderately positive,	++
	R	10	1.5	+	tr	0	+	+	±	Strongly positive,	++++
70	R	5	1.5	+	+	tr	+	+	±	Weakly positive,	+
	R	10	1.5	+	+	0	+	+	±	Moderately positive,	++

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

TEST 4

With Serums 51 to 70, inclusive, first incubation was done in the refrigerator at a temperature of 10 C. for 5 hours and 10 hours. After having added the sensitized blood corpuscles the tubes were placed in the incubator for 2 hours and the results were read from 3-5 hours after the blood corpuscles had been added.

Table 4 shows the results obtained with Serums 51 to 70, inclusive. Serums 51 to 60, inclusive, gave negative results with both methods. Serums 61 to 70, inclusive, gave positive results, and the results were stronger positive after 10-hour first incubation than they were after 5-hour first incubation. The most marked differences were obtained with Serums 64, 65, 66, 69, and 70. All of these serums gave twice as strongly positive results with 10-hour incubation as they did with 5-hour incubation.

TEST 5

With Serums 71 to 80, inclusive, first incubation was done in the refrigerator at a temperature of 10 C. for 5 hours and at room temperature of 21 C. for 5 hours. After having added the sensitized blood corpuscles all tubes were put in the incubator at a temperature of 37 C. for 2 hours and the results were read about 3 hours after the corpuscles had been added.

TABLE 5

FIVE HOUR INCUBATION AT 10 C. COMPARED WITH FIVE HOUR INCUBATION AT 21 C.

Number	Incubation Temperature, C.	Incubation Time, Hours	Amboceptor per Tube Unit	Readings: Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results	
71	10	5	1.5	+	+	tr	+	+	±	Weakly positive,	+
	21	5	1.5	+	+	±	+	+	±	Negative	—
72	10	5	1.5	+	0	0	+	+	±	Strongly positive,	+++++
	21	5	1.5	+	+	tr	+	+	±	Weakly positive,	+
73	10	5	1.5	+	0	0	+	+	±	Strongly positive,	+++++
	21	5	1.5	+	±	0	+	+	±	Strongly positive,	+++
74	10	5	1.5	+	±	0	+	+	±	Strongly positive,	+++
	21	5	1.5	+	+	tr	+	+	±	Weakly positive,	+
75	10	5	1.5	+	+	0	+	+	±	Moderately positive,	++
	21	5	1.5	+	+	±?	+	+	±	Faintly positive,	±
76	10	5	1.5	+	±	0	+	+	±	Strongly positive,	+++
	21	5	1.5	+	+	tr	+	+	±	Weakly positive,	+
77	10	5	1.5	±	0	0	+	+	±	Strongly positive,	++++++
	21	5	1.5	+	tr	0	+	+	±	Strongly positive,	++++
78	10	5	1.5	+	0	0	+	+	±	Strongly positive,	+++++
	21	5	1.5	+	±	0	+	+	±	Strongly positive,	+++
79	10	5	1.5	+	0	0	+	+	±	Strongly positive,	+++++
	21	5	1.5	+	±	0	+	+	±	Strongly positive,	+++
80	10	5	1.5	0	0	0	+	+	±	Strongly positive,	+++++ +++++
	21	5	1.5	+	±	0	+	+	±	Strongly positive,	+++

Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis 50% or less; 0, no hemolysis.

The results obtained with Serums 71 to 80, inclusive, at temperatures of 10 C. and 21 C. are shown in Table 5. With all of these serums complement binding was better at the lower than at the higher temperature.

CONCLUSIONS

Complement binding is better in the incubator at a temperature of 37 C. than in the open water-bath at the same temperature.

Complement binding takes place gradually, many hours being required for its completion.

Complement binding is much better at a temperature of 10 C. than at a temperature of 37 C. or of 21 C.

Perhaps the optimum temperature for complement binding and the length of time necessary for its completion in the Wassermann reaction have not yet been determined.

A STREPTOTHRIX ISOLATED FROM THE BLOOD OF A PATIENT BITTEN BY A WEASEL (STREPTOTHRIX PUTORII)

George F. Dick and Ruth Tunnicliff

From St. Luke's Hospital and the John McCormick Institute for Infectious Diseases, Chicago

The streptothrix described in this article was isolated from the blood of a patient bitten by a weasel. The clinical history, which is typical of rat-bite fever, follows:

A boy, 10 years of age, entered St. Luke's Hospital, Oct. 8, 1917, in the service of Dr. Joseph Brennemann, to whom we are indebted for the privilege of this report. He had been bitten August 10, by a weasel, on the right thumb and left hand. Shortly afterward the wounds were cauterized, and soon they healed. Two weeks later a swelling appeared on the left hand, which became quite large and rather blue in color. The right thumb also became swollen. The axillary glands were enlarged. The patient had fever and general malaise.

On entrance, edema of the lower eyelids was observed. The epitrochlear, axillary and cervical glands were slightly enlarged, and the axillary glands were tender. White blood cell count was 19,000, with 88% polymorphonuclears, 8% large lymphocytes, 4% small lymphocytes. The urine was normal. Wassermann reaction positive. Wassermann reaction of mother and one brother also positive. The temperature was irregular, sometimes rising every day, sometimes at 6 day intervals, the highest point being 106 F. No appetite; vomiting sometimes at the onset of attacks of fever. With each paroxysm, which generally lasted 1 day, edema of the lower eyelids appeared; also an eruption, chiefly on the face and to a slight extent on the chest, of bluish red, sharply defined macules, varying in size from 2-3 cm.

At the onset of the third attack in the hospital, the previous one having been extremely severe, the patient received a small dose of neosalvarsan. This and the following attacks were very mild. The patient was discharged Jan. 1, 1918, in good condition, although the temperature still rose, at times to 101 F.

A point of interest is that another boy, helping to rescue the patient from the weasel, was also bitten, but did not develop the disease. Similar observations have been made in cases of rat-bite fever, two persons being bitten by the same rat, but only one developing rat-bite fever.

Routine blood cultures made during the first paroxysm in the hospital gave on the anaerobic plate a gram-negative bacillus, which did not grow and a gram-positive diplo-streptococcus, which produced hardly any hemolysis, fermented dextrose, lactose and maltose, but not saccharose, inulin and mannite. This coccus was agglutinated by the patient's serum at 1:80, but not by normal serum, and it also gave

Received for publication March 12, 1918.

indications of fixing complement. The coccus was not pathogenic for rabbits or guinea-pigs. Cocci were not found in other blood cultures.

Blood for cultures was drawn again, about 6 hours after the onset of the second attack in the hospital, at the height of the paroxysm, the temperature being 106 F. Cultures were made on plain agar plates, also shake cultures, and in ascitic broth, ascitic fluid and on Loeffler's blood serum slants. In 48-72 hours three to four colonies appeared on the 4 agar plates; the other cultures remained sterile. The colonies were grayish-white, pinpoint in size and regular in outline.

Some of the blood collected in citrate solution was examined with dark field illumination. No spirochetes were observed, but three immobile filaments, from 8 to 20 microns in length and possibly some beaded forms were seen. Blood smears stained with the Giemsa stain and with iron hematoxylin did not show any organisms, but one stained with warm carbol-gentian-violet showed two filaments 8 and 10 microns long, slightly irregular and beaded. They were found only after a long search. According to Tileston[1] the filaments described by him in the blood of his case of rat-bite fever were most abundant early in the paroxysm and become scarcer in each succeeding attack. This may account for the few organisms observed in this specimen of blood.

We were unable to find spirochetes in the blood, urine, eye or cutaneous lesions of the patient. Whole blood, drawn during a paroxysm, was injected into guinea-pigs, but failed to produce any fever. No spirochetes were found in their blood.

The isolated organism is nonmotile, slender, curved, and branching, varying a good deal in length, with bacillary and coccal forms. Branching is most marked in young cultures. Ball and club-shaped terminal swellings are observed, and pleomorphism is marked. When first isolated mostly short forms were seen, later filaments were common, but none of great length or wavyness. The organism is gram-negative, stains with ordinary stains, and is not acid fast.

A facultative anaerobe, it grows better under aerobic conditions. Growth generally occurs in 24-48 hours on blood agar and Loeffler's blood serum as discrete, colorless or grayish-white, pinpoint circular, elevated colonies with sharp margins. At first they appear dull, later become glistening. The medium is not changed. Growth in dextrose blood agar is more profuse and grayish-yellow in color. The growth in ascitic broth is slight, but whitish and flocculent. Growth occurs also in inulin, salicin, maltose, mannite, saccharose, raffinose, lactose and dextrose broths to which ascitic fluid is added (1:4), but there is no change in reaction. There is slight growth only in plain and dextrose broth and milk; on plain and dextrose agar a yellowish-white, glistening growth develops.

The organism is killed by exposure to 57 C. for half an hour.

[1] Jour. Am. Med. Assn., 1916, 66, p. 995.

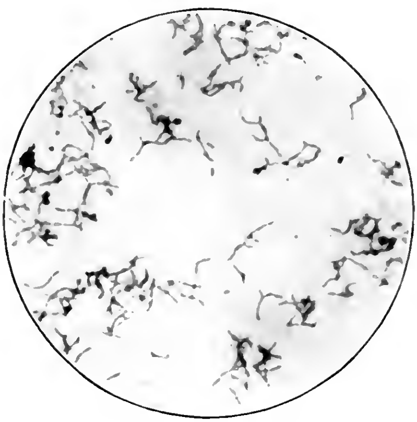
When first isolated it died out quickly and grew with difficulty. After growth was established it remained viable at 37 C. for several weeks and grew more profusely.

Two guinea-pigs were inoculated intraperitoneally with the growth on 3 blood-agar slants. One died on the 6th day after injection, but no lesions were found. The cultures from the blood and kidney were sterile. The other guinea-pig was ill on the 6th day, but recovered. There was no rise in temperature in either animal.

Two young white rats were inoculated intraperitoneally with the growth on 3 blood agar slants. One died on the 3rd day, the other on the 5th day after injection. No lesions were found, and the cultures from the blood and kidneys were sterile. A wild rat was injected with the growth on one slant without any effect.

The blood of each of these animals was examined daily with dark field illumination; but no organisms were observed.

After the streptothrix had been isolated several weeks, the growth on 2 blood-agar slants was inoculated intraperitoneally into 2 white mice, without result.



Twenty-four-hour growth on blood agar. Carbol-gentian-violet. × 1,000.

Between two attacks the patient's blood showed a slight increase in opsonins for the streptothrix. The point of opsonic extinction for normal blood was 1 : 15; for the patient's blood 1 : 30; during an attack the point of extinction was 1 : 60. There was no agglutination with normal serum, but agglutination at 1 : 20 with the patient's serum. The serum of the patient in the presence of the organism fixed complement, and as stated the Wassermann reaction was positive. Fixation tests with Streptothrix muris-ratti isolated from a rat was negative. Soon after neosalvarsan was given the serum no longer fixed complement.

The serum of 7 rats with bronchopneumonia gave complement fixation with this streptothrix in three instances, with 4 strains of Streptothrix muris-ratti in three instances, and with both the organisms in one instance. All the strains of Streptothrix muris-ratti were isolated from the lungs of rats with bronchopneumonia.

Nixon[2] observed that the symptoms of rat-bite fever may be produced by the bite of another animal besides the rat. His patient, a ratter, was bitten by a ferret. He cites another case in his neighborhood, of a man bitten by a ferret, showing the same symptoms. The disease described by Schottmüller[3] as following the bite of a South African squirrel was a pyemia, with purulent skin lesions.

Futaki,[4] Kaneko,[5] Ido,[6] and Kitagawa,[7] have found spirochetes — Spirochaeta morsus-muris—in blood films, skin lesions, lymph glands, kidneys and suprarenals of patients with rat-bite fever in Japan. Similar spirochetes have been found by them in the blood of mice and other animals inoculated with human material. The spirochete has been grown in one generation, from the blood of an infected animal. In Germany and America, a streptothrix—Streptothrix-muris-ratti—has been isolated from the blood of 3 patients with rat-bite fever (Schottmüller, Blake,[8] and Litterer[9]) and observed in the fresh blood in a fourth case by Tileston.

Middleton,[10] in England isolated from a case of rat-bite fever a diplococcus from an anaerobic gelatin blood culture, but did not study its cultural characteristics. Recently Douglas, Colebrook and Fleming[11] studied a case of rat-bite fever and were unable to grow any streptothrices or spirochetes in aerobic or anaerobic blood cultures in simple broth or broth plus trypsin. This is not strange as neither organism as a rule grows in these mediums. They, however, isolated a streptococcus pyogenes from one enlarged gland. On account of the patient's serum agglutinating this organism at 1:60 and as the patient recovered after receiving a vaccine, they regard the condition as due to the streptococcus.

[2] Brit. Med. Jour., 1914, 2, p. 629.
[3] Dermat. Wchnschr., 1914, 58, Supplement, p. 77.
[4] Jour. Exper. Med., 1916, 23, p. 249; 1917, 25, p. 33.
[5] Ibid., 1917, 26, p. 363.
[6] Ibid., 1917, 26, p. 377.
[7] Arch. Int. Med., 1917, 20, p. 317.
[8] Jour. Exper. Med., 1916, 23, p. 39.
[9] Jour. Tenn. State Med. Assoc., 1917, 10, p. 310.
[10] Lancet, 1910, 1, p. 1618.
[11] Ibid., 1918, 1, p. 253.

The streptothrix isolated from the blood of the boy bitten by a weasel differs from Streptothrix taraxeri cepapi obtained by Schottmüller[3] from the pus of the skin lesions and the blood of a patient, bitten by a South African squirrel, in being gram-negative. Schottmüller's streptothrix died before its cultural characteristics were determined. Culturally the weasel streptothrix differs from Streptothrix muris-ratti isolated by Schottmüller, Blake, and Litterer, from the blood of patients with rat-bite fever and also from a similar streptothrix isolated from bronchopneumonia of rats by Tunnicliff,[12] in growing not only more profusely but also in growing on plain and dextrose agar and in plain and dextrose broth and milk; it differs further in not forming long and wavy filaments. On account of these differences, we suggest naming this organism Streptothrix putorii.

CONCLUSIONS

A streptothrix—Streptothrix putorii—has been isolated from the blood of a patient bitten by a weasel.

The clinical picture was similar to that of a rat-bite fever, but the streptothrix differs both morphologically and culturally from the organism associated with that disease, Sterptothrix muris-ratti.

[12] Jour. Infect. Dis., 1916, 19, p. 767.

SIMULTANEOUS ACID AND ALKALINE BACTERIAL FERMENTATIONS FROM DEXTROSE AND THE SALTS OF ORGANIC ACIDS RESPECTIVELY

S. HENRY AYERS AND PHILIP RUPP

From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.

I. AN EXPLANATION OF REVERSION OF REACTION OF CULTURE MEDIUMS BY ORGANISMS OF THE COLON-AEROGENES GROUP

INTRODUCTION

Acid and alkaline bacterial fermentations, as they are usually conceived, involve the splitting up of carbohydrates or similar carbon-containing substances and protein or its decomposition products, respectively. There is no doubt that acids are produced from sugars and undoubtedly ammonia is formed by many bacteria as an end product of protein decomposition; nevertheless an alkaline reaction is not necessarily due to ammonia or alkaline basic decomposition products from protein.

It is strange that the many investigators who have noted alkaline fermentations did not attempt to explain these fermentations on the basis of something other than ammonia, which their figures often showed was not present in amounts sufficiently large to account for the total alkalinity. In some cases the alkaline reaction has been accounted for by either ammonia or alkaline basic substances from protein decomposition perhaps in an attempt to make up the discrepancy between the ammonia actually found and the alkaline reaction.

The fact that the salts of many organic acids may be converted into alkaline carbonates through bacterial agencies has long been recognized but the significance of that type of fermentation seems to have been overlooked. As early as 1878 Hoppe-Seyler[1] found that calcium tartrate and citrate with putrid fibrin gave calcium carbonate as one of the end products of the fermentation. A little later he found that calcium bicarbonate and hydrogen resulted from the fermentation of calcium formate and mud. Maassen[2] worked with 52 varieties

Received for publication March 12, 1918.
Published by permission of the Secretary of Agriculture.

[1] Ztschr. Physiol. Chem., 1879, 2, p. 1.
[2] Arb. a. d. k. Gsndhtsamte, 1895, 12, p. 340.

of bacteria and the sodium salts of 21 organic acids and found that carbonates were formed from the organic salts. It was found by Pakes and Jollyman,[3] who studied the bacterial decomposition of formic acid, that sodium formate could be split up by B. coli into sodium bicarbonate and hydrogen. The same decomposition was also noted by Harden.[4]

From the studies with the same type of organism, Grey[5] believes that calcium formate may split into calcium carbonate, carbon dioxid and hydrogen. Emmerling[6] also states that calcium carbonate could be formed by the bacterial fermentation of calcium tartrate. It has been shown by Omelianski[7] that potassium carbonate is formed by the fermentation of potassium acetate and that calcium carbonate resulted from the fermentation of calcium butyrate. Other organic acid salts besides those already mentioned can be converted into carbonates as is shown by the work of Gingingham.[8] He isolated from soil bacteria which could convert calcium lactate into calcium carbonate when driven to it by lack of other food. Evidently bacteria which are capable of converting the salts of organic acids into carbonates are common in soil, for Temple[9] found that a solution of 1 gm. of potassium dibasic phosphate in 1,000 c.c. of water containing sodium citrate, potassium citrate or sodium potassium tartrate turned alkaline on incubation when previously inoculated with soil. He made the following statement:

"It is clear that soil bacteria can use the sodium or potassium salts of organic acids in such a way as to have one of the products sodium or potassium carbonate."

No attempt will be made to cite all the references in the literature to the bacterial fermentations of salts of organic acids, as there have been many pieces of work reviewed in which other organic acids were formed during the fermentation of a given organic acid salt.

Our attention has been focused on the alkaline fermentation of organic acid salts through an extensive study of the alkaline-forming group of bacteria. In a preliminary paper[10] we called attention to the fact that the alkaline reaction in milk produced by the alkaline-forming group of bacteria was due to the fermentation of salts of citric acid and a production of alkaline carbonates. It was also pointed out that that group of bacteria could ferment the sodium salts of numerous organic acids and convert them into alkaline carbonates. During some later work on the alkali-forming bacteria it was found that some of them could ferment dextrose and it therefore seemed probable that two fermentations, one acid (fermentation of sugar), and the other

[3] Jour. Chem. Soc. (Lond.), 1801, 79, p. 386.
[4] Ibid., p. 610.
[5] Proc. Roy. Soc. Lond., 1914, B, 87, p. 461.
[6] Centralbl. f. Bakteriol., 1908, II 21, p. 317.
[7] Ibid., 15, p. 673.
[8] Jour. Agr. Sci., 1911, 4, p. 145.
[9] Ga. Agr. Exper. Stat., 1914, Bul. 103.
[10] Ayers, S. H., and Rupp, Philip: Science, 1915, 42, p. 318.

alkaline (fermentation of organic acid salts) could progress simultaneously.

Knowing that the organisms of the colon type could utilize carbon from organic acid salts and cause an alkaline reaction it seemed evident that the reversion of reaction in culture mediums by organisms of the colon-aerogenes group as described by Clark and Lubs[11] could be explained by a simultaneous acid and alkaline fermentation. In their first work on the differentiation of the colon-aerogenes group by means of indicators Clark and Lubs[11] used a medium containing 0.5% potassium dibasic phosphate, 0.5% dextrose, 0.5% peptone and distilled water. They found that after five days' incubation at 30 C. cultures of B. coli which gave a low gas ratio $\frac{Co_2}{H} = \frac{1}{1}$ showed a high hydrogen-ion concentration and gave a red color with methyl-red. On the other hand, cultures of B. aerogenes which gave a high gas ratio $\frac{Co_2}{H} = \frac{2}{1}$ showed a low hydrogen-ion concentration and were yellow to methyl-red. It was pointed out by Clark and Lubs that when the sugar content and "regulator" is propertly adjusted the colon type scon reach a high limiting hydrogen-ion concentration which remains constant while the aerogenes type exhausts the sugar before the limiting hydrogen-ion concentration is reached, a reversion in reaction takes place and the medium becomes more alkaline.

It may be assumed off hand that the reversion in reaction is due to the production of ammonia through decomposition of the peptone, although Clark and Lubs pointed out that it should not be assumed that the reversion is due solely to an ammonia production.

In our first experiments it was found that the reversion took place in a synthetic medium in which no ammonia could be liberated from the decomposition of peptone.

THE REVERSION IN A MEDIUM CONTAINING SODIUM-AMMONIUM-PHOSPHATE AS A SOURCE OF NITROGEN

Believing that the reversion by members of the colon-aerogenes group was not due to ammonia resulting from the decomposition of peptone a synthetic medium was prepared. Sodium-ammonium phosphate was used as a source of nitrogen, as that salt had been extensively used in work with the alkali-forming group of bacteria. The composition of the medium was as follows: sodium-ammonium-phosphate 3.6 gm., potassium-acid-phosphate 1.2 gm., dextrose 5 gm., and 1,000 c c distilled water.

[11] Jour. Infect. Dis., 1915, 17, p. 160.

Throughout these experiments we have used a low gas ratio culture termed Fg and a high ratio culture termed Ze. These cultures were supplied to us through the kindness of Mr. Rogers of this laboratory. For convenience Culture Fg is considered B. coli and the Culture Ze, B. aerogenes.

When grown in the very simple sodium-ammonium-phosphate medium it was found that a reversion of reaction occurred with Culture Ze and not with Fg. The hydrogen-ion change in each culture is seen in Figure 1. The B. coli Culture Fg went progressively acid reaching the region of P_H 4.8 while the B. aerogenes Culture Ze reached P_H 5.5 then went progressively alkaline so that on the 6th day it had reached P_H 6.3. These cultures could be differentiated on the 2nd day by methyl-red as Fg had reached P_H 4.9 while Ze was in the region of P_H 5.9.

It was evident from these results that it was possible to get a reversion in a medium free from peptone, which strengthened our opinion that the reversion in a peptone medium was not due to the production of ammonia. The reversion in the sodium-ammonium-phosphate medium could be due only to the formation of bicarbonates or carbonates from the salts of the organic acids produced through the fermentation of the dextrose. Any ammonia liberated from the sodium-ammonium-phosphate through bacterial action would either be taken up immediately by the acid produced in the fermentation of the sugar, in which case it would form an ammonium salt of the acid which would be converted into bicarbonate or carbonate, or it would combine with the acid phosphate to form again sodium or potassium-ammonium-phosphate.

While this work was in progress Clark and Lubs[12] published a paper in which they showed that a reversion took place in a medium containing acid-ammonium-phosphate as a source of nitrogen. They lowered the content of total nitrogen to a point at which its participation in any form in changes of reaction of the medium would become insignificant and showed that an extensive reversion of reaction took place under such conditions. They therefore concluded that in such cases it would be impossible to attribute the reversion to the liberation of ammonia.

The reversion of the reaction is obviously not due to the production of ammonia and as will be shown later is the result of a simultaneous acid and alkaline fermentation in which the salts of the organic acids produced from the sugar are converted into bicarbonates or carbonates.

Simultaneous Acid and Alkali Fermentations in a Sodium-Ammonium-Phosphate Medium

The composition of sodium-ammonium-phosphate medium has been given in this paper. Two series, of 6 flasks each, of this medium were prepared and 6 of the flasks were inoculated with a B. coli Culture Fg and the other 6 flasks

[12] Jour. Biol. Chem., 1917, 30, p. 209.

with an B. aerogenes Culture Ze. Each flask contained 990 c c of medium and was inoculated by adding 10 c c of a fresh culture in the same medium which had been incubated at 30 C. for 24 hours. The flasks were then incubated at 30 C. and one flask containing Fg and one containing Ze were examined for sugar, volatile acids, and its hydrogen-ion concentration after 1, 2, 3, 4, 8, and 14 days' incubation.

The dextrose was determined as cuprous oxid by the official method of Munson and Walker.[13] The quantity of formic acid in the medium was found by experiment not to interfere with the determination of dextrose.

The method for the determination of the volatile acids was as follows: 800 c c of the medium were distilled with phosphoric acid until 700 c c had passed over. The residue was then distilled with steam in a 500 c c flask until a 100 c c of the distillate required less than 0.5 c c of N/10 caustic soda solution for neutralization. The total distillate was evaporated to dryness, dissolved in 100 c c distilled water, filtered, and diluted to 200 c c. The formic acid was determined by oxidation with mercuric chlorid according to the method of Franzen and Eggers[14] and the acetic acid was found by difference.

TABLE 1

FERMENTATION OF DEXTROSE IN A SODIUM-AMMONIUM-PHOSPHATE MEDIUM

	Culture—Fg (Low Ratio)					Culture—Ze (High Ratio)				
Days	P_H	Grams of Dextrose Fermented	C C of N 10 Volatile Acid	Grams of Formic Acid	Grams of Acetic Acid	P_H	Grams of Dextrose Fermented	C C of N/10 Volatile Acid	Grams of Formic Acid	Grams of Acetic Acid
1	5.6	0.9340	114.55	.3245	.2644	6.0	1.8380	103.98	.3098	.2201
2	5.0	1.3080	118.50	.3249	.2876	5.8	3.4980	104.71	.3289	.1998
3	5.0	1.3500	120.88	.3285	.2973	5.7	4.5920	95.90	.3079	.1715
4	4.9	1.4480	118.65	.3194	.2959	6.1	4.6200	67.50	.1911	.1560
8	4.8	1.5280	125.75	.3285	.3265	6.5	4.6420	46.98	.0429	.2261
14	4.8	1.5040	127.13	.3421	.3170	6.5	4.6520	71.03	.0046	.4204
Control Flask 1,000 C C	6.9	Dextrose Grams 4.8240	2.79	.0067	.0080	6.9	Dextrose Grams 4.8080	2.62	.0063	.0073

If, as was suspected, a simultaneous acid and alkaline fermentation took place, then as the fermentation progressed the organic acids should not increase in a constant proportion to the amount of dextrose fermented. An examination of Table 1 shows that such was the case. With Culture Fg (B. coli) the reaction reached P_H 4.8 on the 8th day and remained constant. It will be noted, however, that the volatile acid did not increase in proportion to the amount of sugar fermented, although it followed much more closely than in the case of Culture Ze (B. aerogenes).

In the colon series the amount of formic acid remained fairly constant while the acetic acid increased slightly. With the aerogenes

[13] U. S. Dept. Agr., Bur. Chem. Bul., 1908, 107.

[14] Jour. Prakt. Chem., 1911, 83, p. 323.

culture the hydrogen-ion concentration increased to P_H 5.7 on the 3rd day, then a reversion in reaction took place and on the 8th day the reaction had reached its lowest point, P_H 6.5. While with Fg approximately 1.5 gm. of sugar were used out of a total of about 4.8 gm., Culture Ze utilized about 3.5 gm. within 48 hours and at the end of 72 hours about 4.6 out of a total of about 4.8 gm. were fermented. With this rapid fermentation of sugar it is seen in the table that the amount of total volatile acid decreased after 48 hours' incubation. In

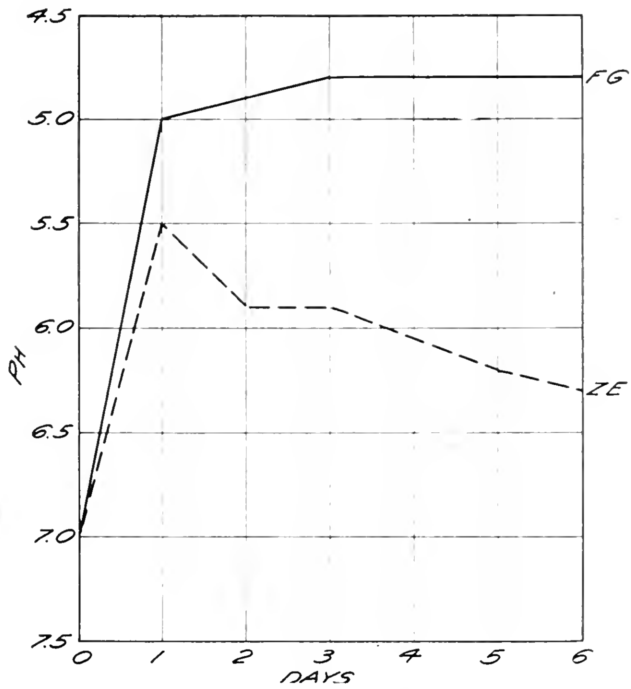


Fig. 1.—The reversion of reaction in a sodium-ammonium-phosphate-dextrose medium by organisms of the colon-aerogenes group.

fact about twice as much dextrose was used after the 2nd day incubation as after 1 day and yet the total volatile acid increased only from 103.98 to 104.71 c c. It is of further interest to observe that the amount of formic acid began to decrease on the 3rd day and there was only a trace left on the 14th day. The acetic acid decreased slightly at first but increased markedly between the 8th and 14th days. This will be discussed later.

The simultaneous fermentation of the sugar and the salts of the organic volatile acids is best shown in Figure 2. In the graph the amount of sugar fermented is plotted on a different scale from the amount of volatile acid but the curves can be compared. It is evident that if the volatile acid was not utilized by the bacteria then it would bear a definite ratio to the amount of sugar fermented. In the case of the B. coli Culture Fg it may be seen from the curves that this is true to a large extent. During the first 48 hours, however, there was

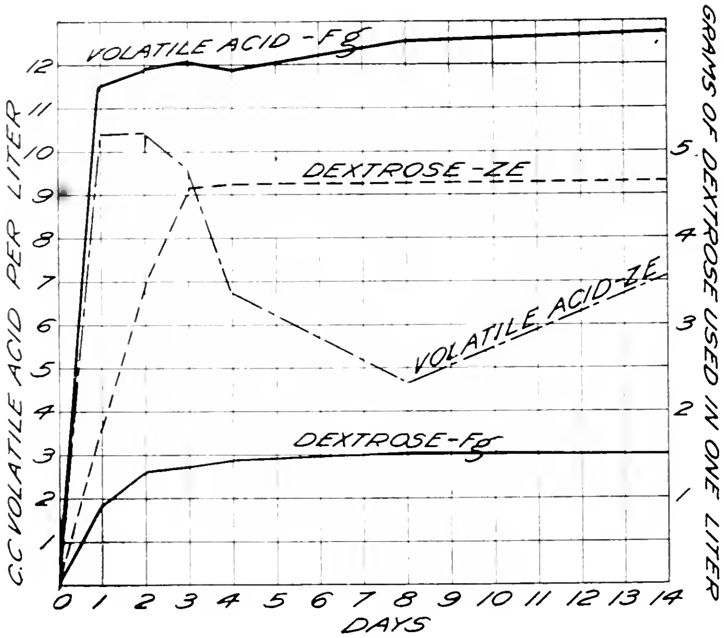


Fig. 2.—Dextrose fermented and amount of volatile acid produced by cultures Fg and Ze.

considerable difference in the ratio, showing that some of the volatile acids was used with the sugar.

B. coli in a sodium-ammonium-phosphate medium soon reaches its highest hydrogen-ion concentration with the fermentation of a relatively small amount of sugar and when this high acid reaction is reached the sugar fermentation practically ceases. Until the highest hydrogen-ion concentration is reached there is evidently a simultaneous acid and an alkaline fermentation which must tend to neutralize each other.

Further examination of Figure 2 reveals the fact that the simultaneous fermentation is more strikingly in evidence in fermentations of B. aerogenes Culture Ze. There was a rapid fermentation of the sugar and also the salts of the volatile acids. The curve shows that the total volatile acid on the 2nd day is about the same as on the 1st, and that there is a rapid decrease followed by an increase after the 8th day. In aerogenes fermentations there is a rapid destruction of the dextrose and at the same time a rapid fermentation of the volatile acids resulting from the sugar fermentation. The salts of the organic acids are oxidized to carbonates or bicarbonates which tend to neu-

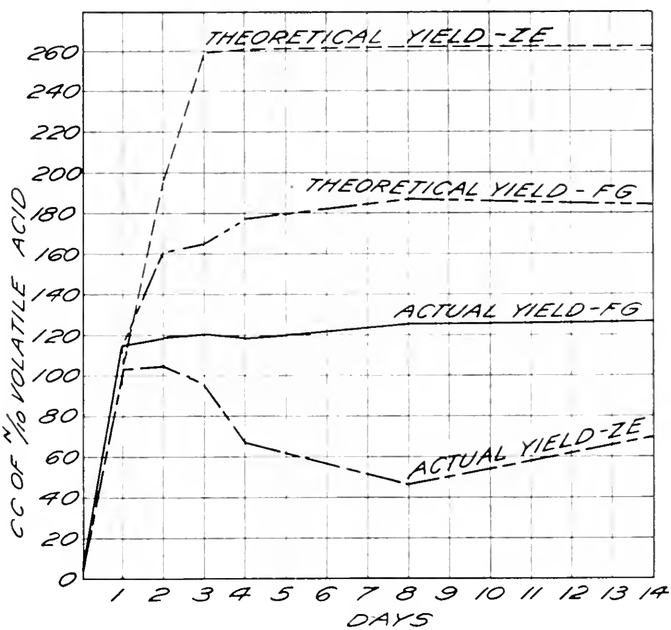


Fig. 3.—Theoretical amount of volatile acid compared with the amount formed.

tralize the acidity, therefore retarding the rise to the highest hydrogen-ion concentration, provided the relation of sugar to buffer is favorable. When the sugar is almost completely used, and it is apparent that it is not entirely used, the alkaline fermentation of the salts of the volatile acids progresses at an increased rate. The increased rate may be due to the partial removal of one source of carbon, the sugar, leaving only the salts of the organic acids or it may be due to the increased amount of the salts of the organic acids in the medium at the time the sugar

is nearly exhausted, or it may be due to both conditions. Since there is no further production of acids the effect of the alkaline fermentation is to cause a reduction in acidity and a reversion of reaction.

Further proof of simultaneous fermentation of sugar and salts of volatile acids is presented in Figure 3. Curves have been plotted to show the amount of volatile acids determined and the theoretical amount which should have been found based on the amount of sugar fermented. As a basis for calculation the proportion of volatile acid present to sugar fermented at the end of 24 hours' incubation was used. It is plain from the graph that much less volatile acid was found than should have been present based on the amount of dextrose fermented. B. aerogenes Culture Ze, it is noted, ferments the salts of the volatile organic acid much more vigorously than the B. coli Culture Fg.

Unfortunately in the determination of the organic acids, the solutions set away and containing the lactic and succinic acids resulting from the bacterial fermentation, molded so that determination of those acids could not be made. It was therefore necessary to repeat the experiment using the same medium in order to determine the formic, acetic, lactic, and succinic acids resulting from the fermentation of the dextrose.

The method of determining lactic and succinic acids was as follows: The residue from the steam distillation of the volatile acids was filtered, neutralized with caustic soda, and evaporated to about 90 c c. This liquid was then acidified with phosphoric acid and extracted with ether for 14 hours in a continuous extracting apparatus. The residue from the ether extract was heated with 100 c c of water and pulverized calcium carbonate on a steam bath for 2 hours, shaking occasionally. The solution was then filtered through an aluminum crucible, washed with hot water, and diluted to 200 c c. In 50 c c of the filtrate the calcium, corresponding to the lactic plus the succinic acids, was determined as oxalate. The remaining 150 c c were evaporated to dryness and the residue taken up with 10 c c of hot water. When cool 90 c c of absolute alcohol were added, the mixture allowed to stand for 2 hours, shaking occasionally, then filtered and washed with 90% alcohol. The filtrate was evaporated to remove the alcohol and calcium of the lactate determined as oxalate. The succinic acid was found by difference.

If, as in the former experiment, the results were plotted and compared, the P_H values, the dextrose fermented and the formic and the acetic found would be practically identical. This fact served as an excellent check on the experiment and showed that fermentation under controlled conditions progressed at a very definite rate.

The results, however, are expressed in a different manner in order to bring out some new points and to emphasize further the simultaneous acid and alkaline fermentation. The results in Table 2 show the

amount of dextrose fermented and the per cent. of formic, acetic, lactic and succinic acid based on the amount of sugar fermented after 1, 4, 8, and 14 days' incubation at 30 C. as well as the hydrogen-ion concentration of the medium.

The B. coli Culture Fg used up about 1.5 gm. of dextrose and in 14 days reached a P_H value of 4.8. It is noted that the percentage of formic acid decreased slightly during the first 4 days and then remained nearly constant. There was practically no change in the percentage of acetic, lactic, or succinic acid during the fermentation. It is of interest to observe that although the highest hydrogen-ion concentration of P_H 4.8 was reached on the 4th day the fermentation did not stop although the rate was slower. This is shown by the increase in the amount of dextrose used from the 4th to the 14th day. The results with the B. aerogenes Culture Ze were quite different. This organism rapidly fermented nearly all the sugar and used up nearly all the formic acid. During the first 4 days the acetic acid was also fermented as is shown by the lowered per cent., but it is also seen that the acid increased between the 4th and 14th day of incubation. This increase of acetic acid may be due to the oxidation of alcohol formed during the fermentation or to the action of the Culture Ze on lactic and succinic acid. Our results do not indicate that lactic and succinic acids are used to any great extent by Culture Ze although the results show some decrease in the per cent. It is interesting, however, to observe that only small amounts of those acids were produced in the fermentation by Ze as compared to the amount produced by Fg, which is also

TABLE 2

ORGANIC ACIDS PRODUCED BY FERMENTATION OF DEXTROSE IN A SODIUM-AMMONIUM-PHOSPHATE MEDIUM

Culture	Days	P_H	Grams of Dextrose Fermented	Per Cent. of Acids Based on Amount of Sugar Fermented			
				Formic	Acetic	Lactic	Succinic
Fg (low ratio)	1	5.3	1.2440	29.17	23.21	Lost	Lost
	4	4.8	1.4680	23.32	22.27	16.92	4.66
	8	4.8	1.5080	22.42	22.36	15.25	5.09
	14	4.8	1.5120	22.71	22.65	15.17	5.38
Ze (high ratio)	1	5.9	2.3100	13.29	11.44	2.58	1.68
	4	6.0	4.8120	5.14	3.05	2.28	1.53
	8	6.5	4.8440	1.72	4.19	1.75	1.38
	14	6.5	4.8480	0.21	8.60	0.90	0.45
Control Flask 1,000 C C		6.9	Dextrose 5.0080	Grams of Formic Acid .0076	Grams of Acetic Acid .0139	Grams of Lactic and Succinic Acids .0202	

true of the volatile acids formic and acetic. It seems probable that the salts of the organic acids are fermented almost as rapidly as produced, thereby making it impossible to determine accurately the amount formed during the sugar fermentation.

The acids produced by the fermentation of dextrose in a synthetic medium using a colon and aergones organism can be seen best in Figures 4 and 5, in which the results previously discussed are plotted. After observing the curve for lactic acid and comparing its position with those of formic and acetic acid one is led to wonder at the statement often found in textbooks that the colon bacillus forms lactic acid from sugar with small amounts of volatile acids. This evidently is

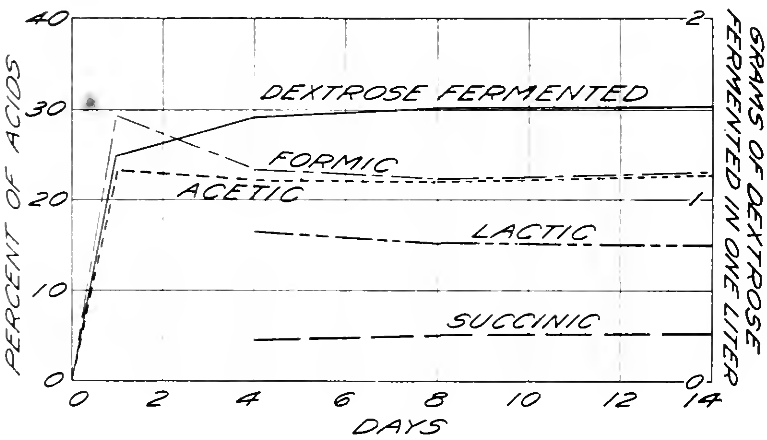


Fig. 4.—Per cent. of various organic acids formed from dextrose by a coli culture.

not true for the fermentation of dextrose in a synthetic medium such as was used in our experiment. The end products of a sugar fermentation, however, vary in accordance with the composition of the medium and the period of incubation.

THE EFFECT ON REVERSION OF REACTION OF THE ADDITION OF SODIUM SALTS OF ORGANIC ACIDS TO THE CULTURE MEDIUM

To this point we have shown that when dextrose is fermented in a medium containing sodium-ammonium-phosphate as a source of nitrogen, the simultaneous fermentation may take place with the colon organism to a slight extent while it is actively produced by the aerogenes type. In this fermentation the dextrose is split into organic

acids which are undoubtedly at once converted into bicarbonates or carbonates.

If this belief is correct then the addition of sodium salts of the acids formed during the fermentation of dextrose to a medium containing dextrose should hasten the reversion of the reaction. We should expect this to assist particularly the aerogenes culture which as has been shown acts most vigorously on the salts of the organic acids.

To determine this effect of the addition of salts of the organic acids 3 mediums were used with the following composition:

No. 1

Sodium-ammonium-phosphate	3.6 gm.
Potassium-acid-phosphate	1.2 gm.
Dextrose	5.0 gm.
Distilled water	1000 c c.

No. 2

Same medium as No. 1, plus 1.2 gm. of the organic acid to be studied. This acid was neutralized with sodium hydrate.

No. 3

Sodium-ammonium-phosphate	3.6 gm.
Potassium-acid-phosphate	1.2 gm.

1.2 gm. of organic acid neutralized with sodium hydrate.

This gave 3 mediums, one with dextrose as a source of carbon, one with both dextrose and the salt of an organic acid, and one with only the salt of the organic acid to supply carbon.

The effect of sodium formate was tried first, as previous results showed that that acid was fermented most readily by the aerogenes culture. Five tubes of each medium were inoculated with the B. coli Culture Fg and the same number with B. aerogenes Culture Ze. One tube of each series was examined for the hydrogen-ion concentration after 1, 2, 3, 5, and 6 days of incubation at 30 C.

It is seen from Figure 6 that both cultures ferment sodium formate and caused a progressive alkaline change when there was no dextrose in the medium. This alkaline change must be due to either bicarbonate or carbonate. The curve of the hydrogen-ion concentration in the dextrose formate medium with coli (Fg) falls below that of the plain dextrose medium. A determination of the increased buffer effect due to the addition of sodium formate showed that it would account for the difference in the curves after the 2nd day of incubation. The difference in hydrogen-ion concentration during the first 48 hours, however, can not be explained by the increased buffer and signifies a

decrease in the hydrogen-ion concentration due to a slight fermentation of the sodium formate.

The effect of the presence of sodium formate on a dextrose fermentation by aerogenes (Ze) is strikingly shown by the curves. When only dextrose was present Ze reached a maximum of P_H 5.2 while in the dextrose-formate medium the maximum was 6.0. Throughout the fermentation the hydrogen-ion concentration of the medium containing dextrose and formate was distinctly lower than that with only dex-

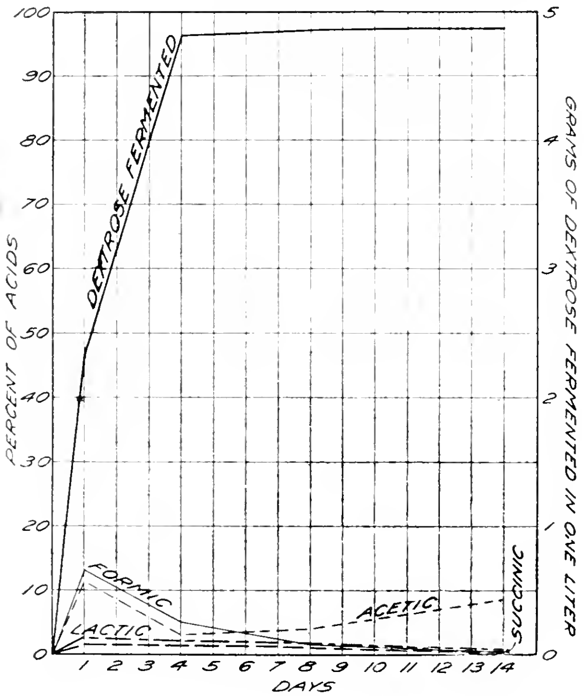


Fig. 5.—Per cent. of organic acids formed from dextrose by an aerogenes culture

trose. The increased buffer effect of the medium due to sodium formate was found to account for only a small part in the lowering of the hydrogen-ion concentration.

When sodium acetate was substituted for sodium formate the effect was much the same as that of the formate. Figure 7 shows, however, that when sodium acetate was the only source of carbon the reaction of the medium did not become so alkaline as with the formate. While the addition of sodium acetate lowered the hydrogen-ion concen-

tration throughout the fermentation its effect was not so marked as that of sodium formate. Here again the increase in the buffer by the addition of sodium acetate plays a part in the lowering of the hydrogen-ion concentration, but it accounts for only a small part of the lowered hydrogen-ion concentration.

The fact that the salts of formic and acetic acid assist in the reversion of reaction by B. aerogenes when added to a dextrose medium together with figures previously presented showing that those acids are

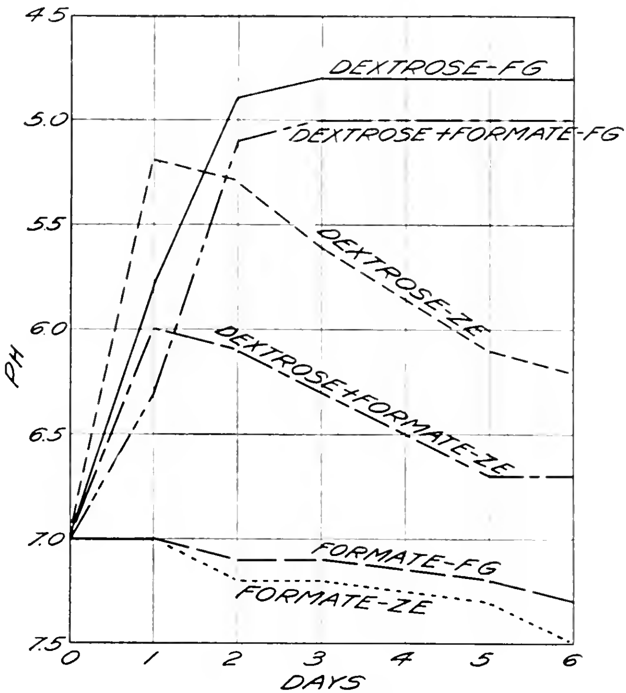


Fig. 6.—Influence of sodium formate on the reversion of reaction by members of the colon-aerogenes group.

used in a dextrose medium in which they were formed from the sugar, seems positive proof that the reversion in reaction with aerogenes cultures is due to their oxidation to alkaline carbonates. We believe that the fermentation of the formate plays the most important part in the reversion since, as has already been shown, it is used more rapidly than the salts of the other acids. The fermentation of the lactate and the succinate do not appear to play any important part although it is

possible that the salts of all the organic acids produced in the fermentation may influence the reversion in reaction as caused by the aerogenes culture.

SIMULTANEOUS ACID AND ALKALINE FERMENTATIONS IN A PEPTONE MEDIUM

From the preceding results it is evident that the reversion in reaction brought about by the aerogenes type of organisms in the synthetic medium used was not due to the production of ammonia. It may be

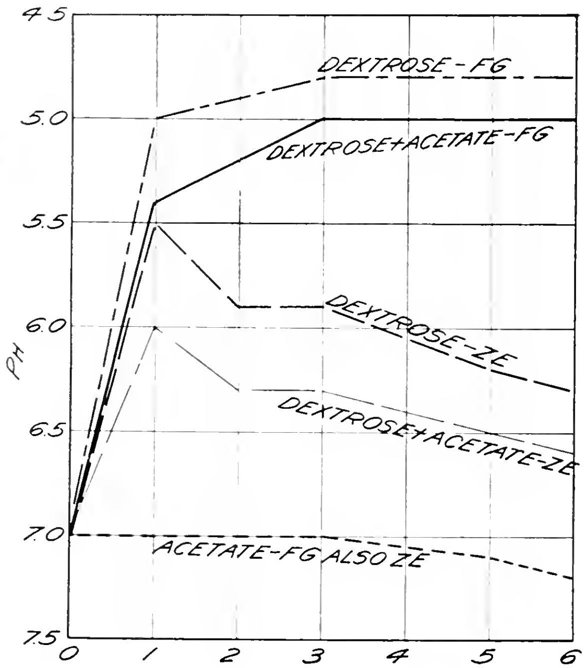


Fig. 7.—Influence of sodium acetate on the reversion of reaction by members of the colon-aerogenes group.

argued, however, that the reversion in a synthetic medium when no free ammonia can be liberated is due to the alkaline fermentation of the salts of the organic acids, but that in a peptone medium the cause of the reversion is the liberation of ammonia as the end-product of protein decomposition. To the bacteriologists the mention of an alkaline reaction in a medium brings to the mind the thoughts of the decomposition of nitrogenous bodies. Thus it is perhaps only natural

that the reversion of reaction in a peptone medium should be assumed to be due to the liberation of ammonia.

We believed, however, that the reversion in a peptone medium was due to an alkaline fermentation of the salts of the organic acids the same as in the synthetic medium and not to ammonia. In order to determine if this were true a study was made of the fermentation in a peptone medium as used by Clark and Lubs.[11]

The medium was made as they directed and comprised the following named ingredients:

Witte's peptone	0.5%
Potassium-acid-phosphate	0.5%
Dextrose	0.5%
Distilled water	

Two series of flasks each of this medium were prepared and one series was inoculated with the B. coli Culture Fg and the other with the B. aerogenes Culture Zc. Each flask contained 990 c c of medium and was inoculated by

TABLE 3

FERMENTATION OF DEXTROSE IN A DEXTROSE-PEPTONE MEDIUM

Culture	Days	P_H	Grams of Dextrose Fermented	Per Cent. of Acids Based on Amount of Sugar Fermented		Milligrams of Ammonia per 100 C C Medium
				Formic	Acetic	
Fg (low ratio)	1	5.1	1.9400	21.69	22.32	4.32
	2	5.0	2.3000	16.60	19.85	4.54
	3	4.9	2.5240	13.25	18.94	2.61
	5	4.9	3.2800	0.12	16.03	2.81
	14	4.9	3.5080	trace	16.14	4.54
Zc (high ratio)	1	5.7	3.8240	12.81	4.81	2.44
	2	5.5	4.9880	9.48	3.27	3.06
	3	6.2	4.9560	7.49	3.23	1.48
	5	6.3	4.9500	0.97	4.74	2.04
	14	6.3	4.9360	trace	12.87	5.12
Control Flask 1,000 C C		6.9	Dextrose 5.0560	Grams of Formic Acid .0029	Grams of Acetic Acid .0171	4.54

adding 10 c c of a culture in the same medium which had been incubated at 30 C. for 24 hours. The flasks were then incubated at 30 C. and one flask of each series was examined for sugar, volatile acids, ammonia, and the hydrogen-ion concentration of the medium after 1, 2, 3, 5, and 14 days. The ammonia was determined by Folin's method and the sugar and volatile acids by methods previously described.

Let us consider first the fermentation by the B. coli Culture Fg. The most interesting feature of this fermentation in a peptone medium is that it varies somewhat from that in a synthetic medium. A comparison of the results in Tables 2 and 3 shows that more than

twice as much sugar was fermented by Fg in a peptone medium as when the source of nitrogen was sodium-ammonium-phosphate. In the peptone medium nearly all the formic acid was used up in 5 days, while in a synthetic medium it was used only to a slight extent. It is of further interest to note that the ammonia was never found in quantities greater than the amounts in the control flasks and so evidently played no part in the reaction.

We wish to call particular attention to the fact that as shown in Table 3 the P_H value reached 4.9 on the 3d day in the colon fermentation and then remained constant in spite of the fact that the amount of dextrose fermented increase from about 2.5 gm. to about 3.5 gm. between the 3d and 14th day of incubation. Evidently this organism does not cease its activities in a peptone-dextrose medium after a certain zone of hydrogen-ion concentration is reached, as believed by Clark and Lubs.[11] It would seem rather that a particular zone of hydrogen-ion concentration is reached and at that point the rate of acid and alkaline fermentations becomes so balanced as to keep the reaction constant, thus allowing further fermentation of the sugar and salts of the organic acids, particularly formic. According to the table the formic acid was used up very largely between the 3d and 14th day of incubation while the reaction remained constant.

In the fermentation of the B. aerogenes Culture Ze there was little difference in the peptone and sodium-phosphate medium, as may be noted by a comparison of the results in Tables 2 and 3. The principal point of interest in this fermentation is the fact that the hydrogen-ion concentration reaches its highest point, 5.5, on the 2nd day and then the medium went progressively alkaline until P_H 6.3 was reached. Keeping that fact in mind the amount of ammonia present in the medium should be observed. Since, with the exception of slight increase between the 5th and the 14th day, there was less ammonia than in the control, it seems evident that the reversion of reaction was not due to ammonia. It should be noted, however, that the formic acid was used up the same as in the synthetic medium.

The prevailing idea has been that an alkaline reaction was evidence of the decomposition of nitrogenous matter with the production of ammonia. This misconception seems to have firmly planted itself in the minds of most bacteriologists who have attempted to explain the reversion of reaction in the coli-aerogenes group. Thus Kligler[15] states: "The progressive change produced in carbohydrate media by

[15] Jour. Bacteriol., 1916, 1, p. 663.

members of the colon typhoid group, excepting certain types, is from alkaline to acid and back. So long as there remains unutilized carbohydrates the acid phase persists. With the complete consumption of the carbohydrates the organism actively attacks the nitrogenous components of the medium, neutralizing the acid and gradually returning to the alkaline phase."

Levine[16] also considers that the alkali is produced from peptone for he says: "It may further be considered that after the limiting hydrogen-ion concentration is reached the organism, if not destroyed, will if capable attack peptones forming alkali. Some of the free acid becomes neutralized and more carbohydrate may be decomposed."

Again we find a similar opinion expressed in the statement of Burton and Rettger[17] writing on the correlations of the colon-aerogenes group: "Following the loss of all the sugar there will occur more or less protein decomposition depending on its availability and thus a certain amount of ammonia production will take place which would account for the alkalinity of some cultures."

So far as our experiments are concerned it is evident that the reversion in reaction by at least one aerogenes culture is due to a simultaneous fermentation of carbohydrate and the organic acid salts produced in its fermentation. The difference between the coli and aerogenes culture is one of rate, the final hydrogen-ion concentration being the result of the rates of the acid and alkaline fermentations. The aerogenes culture ferments the sugar more rapidly than the coli and as the sugar becomes exhausted the rate of fermentation of the organic acid salt becomes greater and the resulting alkaline carbonates cause the reversion in reaction.

It is not strange that the attempts to explain the reversion of reaction in the coli-aerogenes group have been based on the formation of ammonia from protein after the exhaustion of the sugar. Moreover, it is only natural in view of the extensive work by Kendall and his associates on the sparing action of carbohydrates on protein decomposition. In the future, however, the importance of alkaline fermentations of the salts of the organic acids must receive careful consideration.

CONCLUSION

In conclusion we wish to review the facts previously discussed and to offer an explanation of the reversion of reaction in culture mediums by organisms of the colon-aerogenes group.

[16] Jour. Infect. Dis., 1916, 19, p. 773.
[17] Ibid., 1914, 14, p. 411.

It should be understood thoroughly that our results apply to a reversion in reaction in the synthetic medium used in our work and in the peptone medium used by Clark and Lubs.[11] We know that when small amounts of sugar are present and the buffer action of a peptone medium properly adjusted, ammonia may be formed which can produce a reversion in reaction. We believe, however, that in general the reversion in reaction is due to the fermentation of organic acid salts rather than to the formation of ammonia.

The reversion of reaction was produced by an aerogenes culture in a synthetic medium in which ammonia could not cause the reversion.

In a synthetic medium a simultaneous acid and alkaline fermentation took place. While organic acids were produced from the fermentation of the dextrose the salts of those acids were fermented at the same time with the sugar. Sodium formate was found to be utilized to the greatest extent and sodium acetate to a less extent. The salts of lactic and succinic acids were only slightly fermented. Since sodium formate was extensively fermented it seems evident that the alkaline change was due largely to its oxidation to bicarbonates or carbonates.

The addition of sodium salts of formic and acetic acids to a synthetic medium containing dextrose hastened the reversion by an aerogenes organism. Sodium salts of lactic and succinic acids had little or no effect on the reversion of the reaction by the same organism.

In a dextrose-peptone medium as used by Clark and Lubs the reversion in reaction was accompanied with the same simultaneous fermentation of dextrose and salts of the organic acids, particularly the formate. Ammonia played no part in the reversion since there was less ammonia found than in the control uninoculated medium, while the reaction changed from P_H 6.9 to P_H 5.5 and back to P_H 6.3 during a period of 5 days.

Having reviewed briefly the facts brought out in this paper let us now consider the probable explanation of the reversion in reaction by members of the colon-aerogenes group.

1. Dextrose is fermented by both colon and aerogenes organisms and organic acids are formed, which acids combine with the dibasic phosphate-forming salts of the organic acids and acid phosphates, the latter giving an acid reaction.

2. As soon as organic acid salts are formed they are at once fermented simultaneously with the dextrose. Since formic-acid salts are fermented to the greatest extent it is evident that bicarbonates or car-

bonates must be formed; therefore the acid and alkaline fermentations progress simultaneously.

3. It is probable that bicarbonates cause the reversion in reaction, since the formic acid salt is utilized to a much greater extent than the salts of the other organic acids. If the carbonates are formed they would probably be converted into bicarbonates in the presence of the carbon dioxid in the medium.

4. As bicarbonates are produced they react with the acid phosphate in the medium liberating carbon dioxid and forming dibasic phosphate which reacts alkaline and causes a reversion of the reaction. The essential process and the one on which the other adjustments of equilibrium depend is the replacement of a relatively strong organic acid by a relatively weak carbonic acid. Furthermore at the P_H values observed Co_2 must be lost causing an increase in the P_H. For example, equal parts of formic acid and sodium formate in solution have a P_H value of 3.7. When completely oxidized to equal parts of carbonic acid and sodium bicarbonate the P_H value is increased to 6.5. The carbonates lose Co_2 slowly and there is a further increase in the P_H.

5. The amount of formate fermented by an aerogenes culture during the reversion of reaction practically accounts for the change in hydrogen-ion concentration is shown by the following figures. Reference to Table 1 shows that on the 3rd day in the fermentation of Ze the sugar was practically exhausted and the hydrogen-ion concentration had reached its highest point P_H 5.7. At the same time the amount of formic acid present was 0.3079 gm. per liter. On the 8th day a reversion in reaction had taken place and the P_H value was 6.5 while only 0.0429 gm. of formic acid was found. To change the reaction from P_H 5.7 to 6.5 in the medium used 64 c c of N/10 alkali would be required. Calculating the amount of N/10 bicarbonate resulting from the fermentation of the formate during the reversion of the reaction it was found that 0.2650 gm. of formic acid, the amount fermented between the 3rd and the 8th day, corresponded to 57.6 c c of N/10 bicarbonate. Since only 64 c c were required to change the reaction from P_H 5.7 to 6.5 it is evident that the fermentation of the formic acid, in the form of its salts, practically accounted for the reversion in reaction. The alkali fermentation of other organic acid salts probably accounts for the small amount of alkaline change not accounted for by the fermentation of the formate.

6. Colon and aerogenes cultures both produce a simultaneous fermentation of the dextrose and the salts of the organic acids under proper conditions and the reason the aerogenes cultures revert is because they ferment the dextrose and organic acid salts at a different rate from those of the colon cultures.

II. SIMILAR FERMENTATIONS BY ORGANISMS OF THE ALKALI-FORMING GROUP OF BACTERIA

INTRODUCTION

As has been stated, our attention was drawn to the possibility of simultaneous acid and alkaline fermentations, from sugar and the salts of organic acids, respectively, through a study of the alkali-forming group of bacteria. This group comprises those bacteria which produce an alkaline reaction in milk under aerobic conditions without causing visible signs of peptonization. This alkaline reaction is not the result of ammonia formation but, as we[10] have previously reported, is due to the formation of alkaline carbonates resulting from the oxidation of citric acid in milk.

It is quite apparent that the ability of an organism to carry on two types of fermentation at the same time, one acid and the other alkaline from two different sources of carbon, greatly complicates the interpretation of sugar fermentations. These simultaneous fermentations, as has been shown, play an important part in the fermentations of the colon-aerogenes group but, as we shall see, they are not confined to that group.

SIMULTANEOUS ACID AND ALKALINE FERMENTATIONS AMONG THE ALKALI-FORMING GROUP OF BACTERIA

In order to show that the alkali-forming bacteria utilize two sources of carbon simultaneously, 3 different cultures were studied and each was grown in 3 different mediums. In one medium the only source of carbon was dextrose, in another sodium citrate, and in the third the carbon was supplied by both dextrose and sodium citrate. Sodium citrate was used rather than other organic acid salts as the lactate, formate, etc., since these salts would be produced in the fermentation of the dextrose and so complicate the determination of the amount of organic acid salt fermented. Sodium-ammonium-phosphate was used as a source of nitrogen so as to provide a medium of definite composition.

The composition of the mediums was as follows:

No. 1	No. 2	No. 3
Dextrose ....5.0 gm.	Citric acid (neutralized with NaOH) ....2.5 gm.	........2.5 gm.
	.....................5.0 gm.	
Sodium-ammonium-phosphate ....1.5 gm.	.....................1.5 gm.	........1.5 gm.
Potassium chloride....0.2 gm.	.....................0.2 gm.	........0.2 gm.
Distilled water.......1,000 c.c.	...................1,000 c.c.	......1,000 c.c.

Seven flasks of each medium were inoculated and one flask of each was examined after 1, 2, 3, 5, 7, 10, and 14 days of incubation at 30 C. Determinations were made of the hydrogen-ion concentration, the amount of dextrose, and sodium citrate fermented.

For the determination of citric acid, the following reagent was found superior to Deniges reagent which contains too large an excess of sulfuric acid:

Red oxid of mercury........................	50 gm.
Concentrated sulfuric acid.......	100 c c.
Water sufficient to make.........	1,000 c c.

To 50 c c of the medium in a 200 c c graduate flask was added 50 c c of the mercury reagent, the mixture diluted to 200 c c and filtered after 15 minutes. One hundred c c of the filtrate were heated to about 75 C. and a 1% solution of potassium permanganate added, drop by drop, until the precipitate acquired a light buff color, or the shade of a light manila envelope. At first the oxidation was slow but after the addition of 5 or 6 drops of permanganate solution it proceeded rapidly. The solution was heated to boiling, allowed to cool, filtered through a Gooch crucible, and the precipitate washed. It was then dried at 100 C. and weighed. The weight of precipitate multiplied by 0.2708 gave the amount of citric acid.

As may be expected when the course of a fermentation is traced by means of examinations of a number of individual flasks, there was found to be variations in the amount of carbon utilized from day to day. These variations, however, as may be seen in Table 4, were not sufficiently great to interfere seriously with the results. A study of the results reveals the fact that either sodium citrate or dextrose was fermented when present as the only source of carbon and that both were used when in the same medium.

The reactions produced by the fermentation of sodium citrate, dextrose, and both together, is best shown by the hydrogen-ion concentration plotted in Figure 8. Culture 10 fermented sodium citrate and caused a progressive alkaline change in the medium reaching P_H 8.5. With dextrose as a source of carbon an acid fermentation was produced, P_H 4.5 being reached on the 5th day. When both sodium citrate and dextrose were in the medium, it is noted that the acidity increased slightly on the 1st day, then the reaction went progressively alkaline.

When comparing the hydrogen-ion concentration of the dextrose and dextrose and citrate mediums during the course of the fermentation of these alkali-forming bacteria, we must not lose sight of the fact that the buffer actions of the two mediums are entirely different. The addition of sodium citrate greatly increases the buffer action of the medium and therefore lowers the hydrogen-ion concentration as the acid fermentation progresses. From this it is apparent that it is unsafe to assume offhand, that the difference in the hydrogen-ion concentration in the fermentation of dextrose alone and dextrose with

TABLE 4

FERMENTATION OF DEXTROSE AND SODIUM CITRATE BY ALKALI-FORMING BACTERIA

Culture No.	Days	Medium Containing Citrate		Medium Containing Dextrose		Medium Containing Citrate and Dextrose		
		P_H	Milligrams of Citrate Fermented in 100 c c	P_H	Milligrams of Dextrose Fermented in 100 c c	P_H	Milligrams of Citrate Fermented in 100 c c	Milligrams of Dextrose Fermented in 100 c c
Control		7.6	253.3 in 100 c c	7.3	471.2 in 100 c c	7.6	249.3 in 100 c c	448.0 in 100 c c
10	1	7.6	15.1	6.8	43.1	7.3	0.0	2.0
	2	7.8	25.6	6.3	73.4	7.4	0.4	10.7
	3	7.8	24.6	4.6	89.9	7.4	11.0	21.5
	5	8.2	45.8	4.5	97.6	7.7	25.4	26.0
	7	8.2	46.5	4.5	91.6	7.8	42.3	32.0
	10	8.3	56.7	4.6	82.8	7.9	56.7	67.3
	14	8.5	57.7	4.5	94.4	8.0	91.5	67.3
21	1	7.6	0.9	7.0	34.4	7.4	2.3	4.7
	2	7.6	2.8	6.9	40.0	7.3	19.1	16.0
	3	7.7	8.1	6.8	52.4	7.4	35.3	18.0
	5	7.8	14.8	6.3	115.8	7.3	24.0	41.3
	7	7.9	29.1	6.0	78.2	7.4	40.1	48.0
	10	8.2	45.6	6.0	95.6	7.2	47.3	65.3
	14	8.2	42.7	5.4	91.2	7.1	56.3	64.0
110	1	7.6	0.0	6.8	64.4	7.2	0.0	62.6
	2	7.6	1.1	5.9	103.0	7.3	29.3	71.3
	3	7.7	13.2	4.5	108.6	7.4	9.7	18.7
	5	8.0	32.6	4.5	148.0	7.5	12.1	84.4
	7	8.2	48.6	4.5	122.2	7.5	31.8	30.0
	10	8.3	67.9	4.5	97.6	7.3	38.2	115.3
	14	8.5	71.6	4.5	112.2	6.6	68.1	158.0

sodium citrate is due to production of alkaline carbonates. It is easy to measure the buffer action of the sodium citrate and determine what part it plays in reducing the hydrogen-ion concentration if we assume that it is not used up during the fermentation. The buffer action of this salt is, however, complicated by the fact that in the fermentation it is constantly being fermented and its buffer effect consequently lost. Assuming, however, that it remains in a constant amount throughout the fermentation it was found that its buffer action was not anywhere near great enough to give account for the hydrogen-ion concentration determined during the fermentation of dextrose and sodium citrate.

Since the fermentations with Culture 10 did not show any variations throughout the series, we have plotted in Figure 9, the amount of sodium citrate and dextrose fermented and the reaction in terms of P_H values. The curve shows that after 1 day the acidity had increased slightly which correlates with the fact that at that time some dextrose had been fermented but no citrate. On the 2nd day the reaction had started on its alkaline course and some citrate had been fermented. It

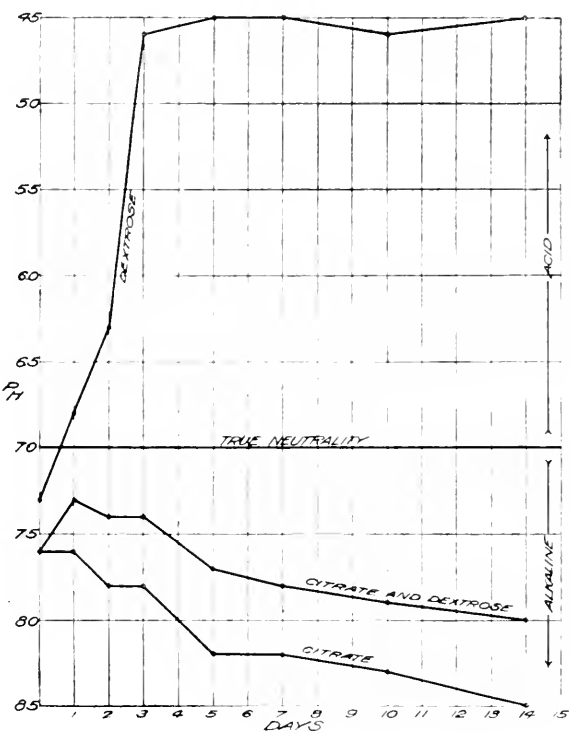


Fig. 8.—Reaction produced by fermentation of dextrose and sodium citrate, separate and together, by Culture 10.

was a small amount, however, and here the possibility must be noted that there may have been some alkaline carbonates formed from the fermentation of organic acid salts produced from the fermentation of the dextrose. This is a probability which exists throughout the experiment. This, however, does not detract from the results which show that the sodium citrate and dextrose were fermented to about the same

extent during the first 11 days while the reaction was constantly going alkaline.

The reaction resulting from the simultaneous acid and alkaline fermentation among the alkali-forming bacteria depends on the rate of fermentation of the sugar and the salts of the organic acids, and the buffer effect of the medium. As has just been seen with Culture 10 and 14 days' incubation 67.3 mg. of dextrose was fermented and

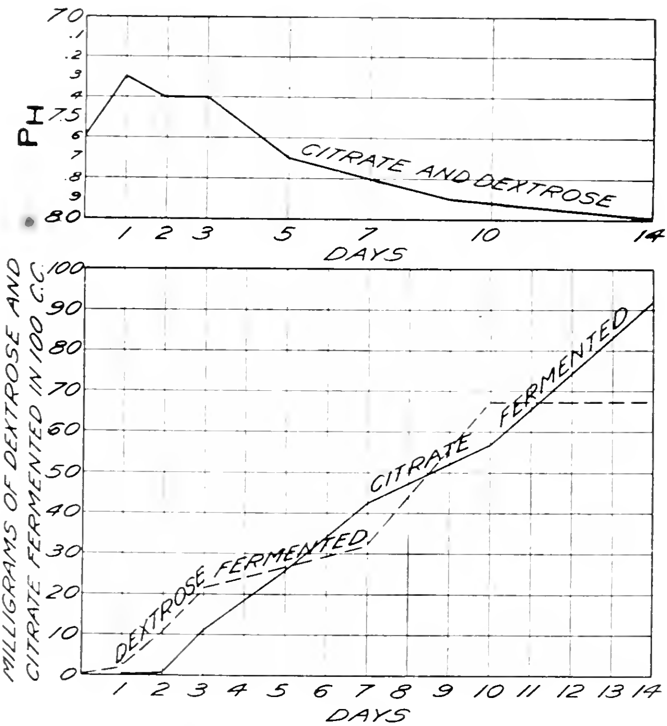


Fig. 9.—Amount of detrose and sodium citrate fermented in same medium by Culture 10 together with the hydrogen ion concentration of the medium.

91.5 mg. of sodium citrate, and as a result the reaction was changed from P_H 7.6 to P_H 8.0 an alkaline change.

Culture 31, on the other hand, fermented 64 mg. of dextrose and 56.3 mg. of sodium citrate when both were present in the same medium, and the hydrogen-ion concentration changed as is shown in Figure 10, from P_H 7.6 to P_H 7.1, a slight acid change. This culture caused an alkaline fermentation with sodium citrate when used as the

only source of carbon. In the dextrose fermentation the type of curve is quite different from that of Culture 10 for the acidity increased slowly and only reached P_H 5.4. In spite of the fact about the same amount of dextrose was fermented by both cultures as reference to Table 4 shows. This points to an active fermentation by Culture 31 of the salts of the organic acids produced in the fermentation of the dextrose.

Fig. 10.—Reaction produced by fermentation of dextrose and sodium citrate, separate and together, by Culture 31.

Culture 110 showed even different results in the fermentation of sodium citrate and dextrose in the same medium. After 14 days 158.0 mg. of dextrose and only 68.1 mg. of citrate were fermented and as a result the reaction changed as is shown in Figure 11, from P_H 7.6 to P_H 6.6, a distinct acid change.

These results have been discussed more fully perhaps than would seem to be warranted at first thought. If, however, these simultaneous fermentations are clearly understood their far-reaching importance will at once be evident.

SIGNIFICANCE OF SIMULTANEOUS ACID AND ALKALINE FERMENTATIONS

Since an organism can ferment sugar and form organic acids and at the same time ferment the salts of the same acids and oxidize them to alkaline carbonates it is plain that these simultaneous fermentations may occur in any medium containing a fermentable sugar. Even in a synthetic medium this may occur if the organism is capable of utilizing

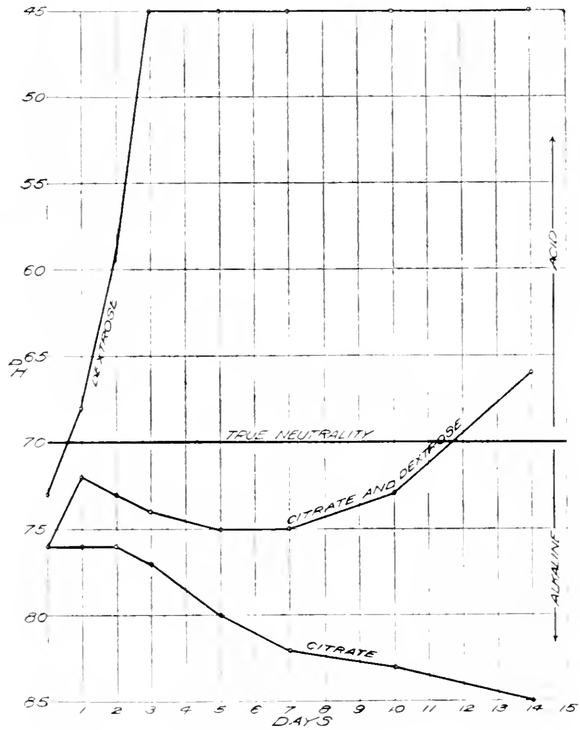


Fig. 11.—Reaction produced by fermentation of dextrose and sodium citrate, separate and together, by Culture 110.

carbon from the salts of the particular organic acids formed in the sugar fermentation. When the source of nitrogen in a medium is some definite substance like sodium-ammonium-phosphate, the hydrogen-ion concentration is a measure of the resultant of the acid and alkaline fermentation and under definite conditions of incubation, and when the sugar and buffer are properly adjusted, the reaction should be a measure of the rate of the two simultaneous fermentations devel-

oping from the primary fermentation of the sugar. In a synthetic medium free from organic acid salts the sugar must of course first be attacked, as there are no organic acid salts present. We may speak therefore, under these conditions of the primary sugar fermentation producing acid and the secondary organic acid salt fermentation producing alkaline carbonates. These terms "primary" and "secondary" are probably correctly used only in a theoretical consideration of the fermentation, since as soon as organic acid salts are produced from the sugar they can be immediately fermented, after which the two fermentations progress simultaneously.

The ordinary mediums contain organic acid salts, for they usually contain beef extract or meat infusion. This is especially true of meat infusions which are freed from sugar by fermentations with B. coli. In such cases not only are there organic acid salts normally present in the meat infusion but in addition those produced through the coli fermentation of the sugar. These sugar-free mediums, as well as sugar broths containing beef extract, have been and are now commonly used in the study of sugar fermentations by bacteria.

When sugar fermentations are conducted in such mediums the reaction may be due to the acid fermentation of the sugar and an alkaline fermentation not only of the salts of the organic acids produced from the sugar but those in the medium before inoculation. These simultaneous fermentations greatly complicate the significance of acid tests for purposes of classification, a fact which has been justly recognized. Thus Rogers, Clark and Davis[18] writing about alkali formation by organisms of the colon group, state: "This property of alkali formation with the subsequent tendency to uncontrolled variation, reduces very materially the value of the titer of sugar broths for diagnostic purposes." Also, Levine[16] after studying the acid production of coli-like bacteria concludes that acid formation should not be given precedence over gas formation in studies on B. coli, for the acid may be masked by a secondary alkali production.

Here we wish to call attention again to the fact that the acid and alkaline fermentations progress simultaneously so long as there is a sufficient quantity of sugar and organic acid salts to be fermented. The alkaline fermentation of the salts of the organic acids must not be considered as a secondary reaction in the sense that it occurs after the sugar is exhausted. This may at times be apparent since in some cases the revision in reaction comes after several days of incubation

[18] Jour. Infect. Dis., 1917, 21, p. 162.

and when it may be reasonably assumed that the sugar is nearly exhausted. In such cases the rate of the alkaline fermentation of the organic acid salts may be accelerated but it is not the beginning of the fermentation. The term "secondary fermentation" therefore should not be applied since it is liable to give a false conception of the true fermentations.

The fact that these simultaneous fermentations complicate and decrease the value of acid determinations in sugar fermentations in ordinary mediums should not discourage the use of sugar fermentations for diagnostic purposes. Rather should it lead to a broader conception of sugar fermentations and the application of the principles of the acid and alkaline fermentations of a sugar, if we may call it such.

As we have stated, under controlled conditions, knowing the ability of bacteria to obtain carbon from sugar and salts of organic acids which can be formed from the sugar, it should be possible to measure the rates of the two fermentations and put them to some practical diagnostic use." As an example, we may mention the "Methyl-red test" devised by Clark and Lubs[11] which operates on the principle of simultaneous fermentations, although they were not clearly understood when the test was made. Further studies of various groups of bacteria based on these fermentations should reveal characteristics of value for diagnostic purposes.

SUMMARY AND CONCLUSIONS

1. Simultaneous acid and alkaline fermentations may be produced by members of the alkali-forming group of bacteria through the fermentation of sugar and salts of organic acids.

2. In a synthetic medium containing dextrose and sodium citrate an acid and alkaline fermentation takes place and the dextrose and citrate are used simultaneously.

3. The rate of fermentation of the dextrose and citrate varies with different cultures.

4. Simultaneous acid and alkaline fermentations, where carbonates are formed from the oxidation of salts of organic acids, may complicate and detract from the value of the determination of acidity in sugar fermentation. Under controlled conditions, however, the simultaneous fermentations can probably be used to considerable advantage for diagnostic purposes.

THE PRECIPITIN REACTION IN THE URINE IN PNEUMONIA

William J. Quigley

From the Memorial Institute for Infectious Diseases, Chicago

Avery and Dochez[1] have shown that a soluble substance is diffused through the medium during growth of pneumococci, that it is elaborated by the growing organism, and not the result of death of the culture. They showed that it is also formed in pneumococcal infection in man, being demonstrable in the urine and blood by precipitation with specific serum. According to a later report[2] the urine of 80 patients, sick with pneumococcus infection (Groups I, II and III) has been examined and specific reactions obtained in 65%. The mortality in this series led to the suggestion that the presence of the reacting substance in the urine is indicative of severe intoxication, and of unfavorable prognostic significance.

I have made study of the sputum for the type of infection, and of the urine for precipitin reactions in 100 cases of lobar pneumonia due to the pneumococcus, occurring in Chicago during the present winter. Cultures from the sputum were made on blood-agar plates or intraperitoneal mouse inoculation made of sputum; the organisms thus obtained were tested for bile solubility and for agglutination by specific serum (Rockefeller Institute). There were 33 instances of infection with pneumococci of Type I, 36 of Type II, 13 of Type III, and 18 of Type IV. The proportions are similar to those reported by Avery, Chickering, Cole and Dochez,[2] from a study of 454 cases with the percentages of 33, 33, 13 and 20, and covering a period of several years. Hence the incidence of type in pneumococcus infections may be quite constant through different seasons and localities. In the group of cases quoted 4% of the pneumococci were of Subgroup II, that is, they were agglutinatable by undiluted Group II serum, but not in dilution of 1:20. I found but 1% of this subgroup.

The urine, and in some instances the blood serum, were tested at 2 or 3 day intervals during the course of the disease for precipitin

Received for publication March 12, 1918.

[1] Proc. Soc. Exper. Biol. and Med., 1916-1917, 14, p. 126.

[2] Avery, Chickering, Cole and Dochez, Monographs of the Rockefeller Institute for Medical Research, 1917, 7, p. 34.

reactions with the 3 types of antipneumococcus serum. Small quantities of urine clarified by centrifugation, were stratified with equal amounts of immune serum in small agglutination tubes, incubated for 1 hour and observed at intervals. The precipitate usually appeared within 15 minutes, if at all; in a few cases only after incubation for ½-1 hour.

Of 82 cases of Types I, II and III, the urine of 67 (81%) showed at some time during the disease a specific precipitin reaction. The strength of the reaction gradually increased during a period of 3 or 4 days, persisted from 2-19 days, and gradually disappeared. It was present during the 3rd week in a few convalescents when discharged from the hospital. There was no regularity as to the period in the disease at which it appeared, nor as to how long it persisted. Reactions were first positive as early as the 2nd day of the illness, and as late as the 21st day during convalescence. They seemed not to be influenced by the crisis.

TABLE 1

RESULTS OBTAINED IN 100 CASES OF LOBAR PNEUMONIA

Types of Pneumonia	Incidence of Types	Specific Precipitin Reaction in Urine		Mortality			
				Positive Precipitin Reaction in Urine		Negative Precipitin Reaction in Urine	
		Positive	Negative	Recovered	Died	Recovered	Died
I	33	24	9	14	10	6	3
II	36	32	4	20	12	3	1
III	13	11	2	10	1	2	0
Total	82						
IV	18						
Total	100	67	15	44	23	11	4

As to mortality, 30 of the 100 patients died. The group mortality was approximately 39%, 36%, 8% and 17%, respectively, for the 4 types. Of the 27 deaths due to Type I, II and III infections, 23 showed a precipitin reaction in the urine; 4 were negative. Of the 67 showing a precipitin reaction 23, or 34%, died. Of the 15 with negative reactions 4, or 27%, died.

It therefore appears that a large percentage of fatal cases of pneumonia have a substance in the urine capable of giving a specific precipitin reaction with antipneumococcus serum, but that the presence of this substance in the urine is not of great unfavorable prognostic value, as the mortality when it could not be demonstrated was only

slightly lower than when it was present. In every instance in which a precipitin reaction occurred it was specific for the serum corresponding to the type of organism present as determined by agglutination of the pneumococcus isolated from the sputum. When this reaction is present in the urine it is an accurate means, and at present the most simple and rapid means of type determination, and is, according to my results, present in 81% of cases due to Type I, II and III infections, although not always sufficiently early to warrant neglect of the sputum examination for the type of infection.

THE EFFECT OF THE CULTURE MEDIUM ON THE AGGLUTINATIVE PROPERTIES OF BACILLUS PARATYPHOSUS B.

Ruth Victorson

From the Department of Hygiene and Bacteriology, University of Chicago

Theoretically, differences in the metabolism of bacteria may affect agglutination in two ways: Either the agglutinogenic substance or the agglutinable substance may be changed. If the agglutinogenic substance be diminished, injection of the bacteria into animals will cause a lowered production of agglutinins; if increased, a serum of higher titer. As a result of diminishing the agglutinable substance, the organisms will not agglutinate even in the presence of a serum of high titer; increasing the agglutinable substance results in the agglutination of the organisms even by a serum poor in agglutinins. The specificity of the agglutination may also be affected.

Many observations have been made on variations in agglutinations due to the growth of bacteria on blood agar or serum broth. Kirstein[1] found that typhoid bacilli, when grown in serum broth, were slightly less readily agglutinated than when grown in ordinary broth. Bordet and Sleeswijk[2] noted that the serum of rabbits inoculated with a plain agar strain of B. pertussis would agglutinate only that strain, while the serum of rabbits immunized with organisms grown on hemoglobin agar would agglutinate both strains. Later Bordet[3] observed that the blood agar strain, if kept for a few years, would not agglutinate with the serum of a rabbit immunized with a freshly isolated strain; in other words, the artificial cultivation of this strain on blood agar brought about the same change in a few years that was noticed on plain agar in a few days. The agglutinable substance in typhoid bacilli is decreased by growth on blood agar according to Sick.[4] The most striking results were obtained by Gay and Claypole[5] who, in working with B. typhosus, found that two successive transfers on 10% rabbit blood agar produced a strain whose serum would agglutinate its own culture and the plain agar strain indiscriminately, but which could not be agglutinated by the serum of a rabbit immunized with the plain agar strain. They advise, therefore, using animals immunized with the blood agar strain for the purpose of diagnosis. Bull and Pritchett,[6] however, were unable to confirm Gay and Claypole's observations altho they made 25 successive transfers on 10% blood agar of 57 strains of typhoid bacilli.

Received for publication Feb. 25, 1918.

1. Ztschr. f. Hyg., u. Infektionskr., 1904, 46, p. 229.
2. Ann. de l'Inst. Pasteur, 1910, 24, p. 476.
3. Jour. State Med., 1913, 21, p. 513.
4. Centralbl. f. Bakteriol., I, O., 1912, 64, p. 111.
5. Arch. Int. Med., 1913, 12, p. 621.
6. Jour. Exper. Med., 1916, 24, p. 35.

Lessened agglutinability in cultures of B. typhosus freshly isolated from the body has been commonly noted, yet Porges and Prantschoff[7] injected a suspension of living typhoid bacilli into the ear vein of a rabbit and found, when they isolated the organism from the gallbladder 35 days later, that it did not agglutinate differently from the original culture which had been kept growing on ordinary media. Sacquépée,[8] however, caused strains of typhoid bacilli to become less readily agglutinated due to growth in collodion sacs in the peritoneal cavities of rats immunized to typhoid. The change was slow, taking 5 months or more and was easily lost by transferring a few times on plain agar. Walker[9] found that cultivation of the typhoid bacillus in its immune serum gave diminished agglutinability, but this decrease was very transitory. Similarly, Moon[10] caused poorly agglutinable strains recently isolated from typhoid patients to have a higher agglutinability by cultivation on agar. Stryker,[11] in studying the reactions of pneumococci, observed that when grown in its immune serum (4 transfers) a pneumococcus showed decreased agglutinability with its homologous serum and less specificity. Normal serum broth and ordinary broth did not, however, affect the reaction of these pneumococci.

The effect of utilizable carbohydrate on bacterial metabolism has been a subject of much interest and possibly, as Kendall[12] has stated, of practical importance. His work showed that the presence of fermentable sugar in considerable amounts in the medium in which bacteria of the colon-typhoid group are grown will reduce the nitrogen content of their bodies one-fifth. Kendall, therefore, concluded that until the exact influence of this variation in nitrogen in terms of immunizing power can be determined, it would be logical to grow bacteria to be used for vaccines in a protein medium free from sugar. Gloessner,[13] in studying the effect of the medium on the agglutinability of bacteria, noted that the presence of sugar had an unfavorable influence on the agglutinogenic properties of B. typhosus and caused a slight decrease in agglutinability. However, as reported by Simonds,[14] the injection of suspensions of killed typhoid bacilli grown for 2 weeks on dextrose agar gave a serum of higher titer than the injection of suspensions of B. typhosus kept on plain agar. He also noted that cultivation on dextrose agar increased the agglutinability of the bacilli. Similarly conflicting observations are made by Sehrwald[15] and Eisenberg[16] concerning the effect of growth of the typhoid, paratyphoid, and dysentery bacilli on potato. The former claimed that the organisms were more readily and rapidly agglutinated than when cultivated on any other medium, but the latter found no variation in the agglutinability of the bacilli.

Changes caused by bactericidal substances have been reported by few observers. Altmann and Rauth[17] by growing B. coli for several generations on "carbol-agar," produced a strain which would not agglutinate with the serum of rabbits immunized with the original strain nor would the serum produced

[7] Centralbl. f. Bakteriol., I, O., 1906, 41, p. 658.
[8] Ann. de l'Inst. Pasteur, 1901, 15, p. 249.
[9] Jour. Path. and Bacteriol., 1902-3, 8, p. 34.
[10] Jour. Infect. Dis., 1911, 8, p. 463.
[11] Jour. Exper. Med., 1916, 24, p. 49.
[12] Boston Med. and Surg. Jour., 1913, 168, p. 825.
[13] Ztschr. f. exper. Path. u. Therap., 1905, 1, p. 640.
[14] Jour. Infect. Dis., 1915, 17, p. 500.
[15] Deutsch. med. Wchnschr., 1905, 31, p. 261.
[16] Centralbl. f. Bakteriol., I, O., 1906, 41, pp. 752 and 823.
[17] Ztschr. f. Immunitätsforsch., Orig., 1910, 7, p. 629.

by this "carbol-agar" organism agglutinate the plain agar strain. This variation was relatively permanent, for a month's passage on plain agar did not make it revert to the original reaction. Staphylococci exposed to corrosive sublimate (0.1%) or to phenol (0.75%), according to Abbott,[18] can produce a serum which will agglutinate the normal agar strain in a dilution of 1:1000. However, these organisms can be agglutinated by their own serums no higher than in a dilution of 1:40 and show no trace of clumping with the serum of normal staphylococci. These bacteria regain their former agglutinating power when transferred on normal mediums 5 or 6 times. The observations of Russ and Chambers[19] that diphtheria bacilli clump when exposed to radium emanations make it seem probable that slight variations in the radium content of the culture medium may bring some variability in agglutination.

That the physical environment may affect the agglutination of the organism is shown by the following observations. Nicolle and Trenel[20] found that cultivating the typhoid bacillus at 42 C. caused a loss in both its agglutinative and agglutinogenic properties. Later Eisenberg[16] caused a reduction in the agglutinability but not in the agglutinogenic properties by growing B. typhosus at this temperature.

With a few exceptions all the observations on this subject agree that variations in the agglutinogenic and agglutinative properties are very difficult to effect and that such changes consist of a slight decrease in the highest dilution to which the organisms are agglutinated rather than the complete loss of the agglutinogenic or agglutinative powers. Such variations are transitory rather than permanent.

The conflicting statements in the literature and the few attempts at producing variations in paratyphoid bacilli made it a matter of interest to study the effect of the medium on the agglutination of B. paratyphosus B. The organism used was a culture isolated from the blood of a paratyphoid fever patient by Irons and Jordan[21] (Para. B. 12). This bacillus has shown constant characteristics during the 5 years it has been kept in stock.

The mediums tested were sugar-free agar as a control medium, 10% rabbit blood agar, Loeffler's serum, 1% dextrose and 1% lactose broth and agar, potato, and Jordan's nonprotein medium to which was added 1.5% agar. The reaction of the sugar mediums and sugar-free agar was 0.5% acid to phenolphthalein. The organism was transferred on these mediums daily for 2 weeks before the first injection into rabbits was made and 44 times before the agglutination tests were tried. To control the factor of individual variations in rabbits, 2 animals of different types were inoculated with each strain of the bacillus.

[18] Jour. Med. Research, 1912, 26, p. 513.
[19] Proc. Roy Soc. Med., 1911-1912, 5, p. 198, Path. Sect.
[20] Ann. de l'Inst. Pasteur, 1902, 16, p. 562.
[21] Jour. Infect. Dis., 1915, 17, p. 234.

Altho Neisser[22] showed that there is no strict parallelism between the number of bacilli injected and the agglutination value produced thereby, yet it seemed better to standardize the suspensions before injecting them into the rabbits, 100 million bacteria being injected each time. These suspensions were made in sterile physiologic salt solution from 24-hour old cultures of the medium tested except in the case of the potato strain. Since the suspensions made directly from this medium contained so many potato particles, the strain was transferred to agar once before injection. The tables containing my results do not include the original agglutinating value of the rabbits since only 2 agglutinated in a 1:20 dilution, and most did not clump the bacteria at all. The rabbits were immunized by injecting first killed and then live suspensions of the bacteria subcutaneously and intraperitoneally. They were bled 10 days after the third intraperitoneal injection.

The macroscopic method of agglutination was used, each strain being tested not only with its homologous serum, but with each heterologous serum also. Instead of merely adding 10 c c of physiologic salt solution to each slant as is ordinarily done, the suspensions for the agglutination tests were made by washing down cultures and then diluting them to a like density (roughly estimated by the microscopic method of standardizing vaccines), thus avoiding any variation in agglutination due to a difference in the number of bacilli present. To each serum dilution (1 c c) the same amount of suspension was added. The tubes were then incubated at 37 C. for 2 hours, placed in the ice-box for 18 hours, and read. To check results I marked the tubes in cipher and did not ascertain their identity until all the results were read and recorded.

Incidentally, it may be observed (1) that the growth on blood agar was almost 3 times as heavy as on ordinary agar and the bacilli showed a pronounced variation in morphology, becoming thicker and larger, more oval shaped, and tending to grow in chains. (2) A similar change was noted on Loeffler's serum, some of the bacilli also forming granules. (3) The growth on the sugar-containing mediums was almost 50% denser than on the sugar-free agar. (4) An effect of potato on paratyphoid bacilli was manifested by the fact that altho the development on potato was slight even in 48 hours, yet return to ordinary agar gave a much heavier growth of bacteria than formerly. (5) At first, cultivation of the organism on nonprotein agar was difficult, but when

[22] Ehrlich: Collected Studies on Immunity, 1906, Chap. 12.

it had become accustomed to this medium about 60% as many bacteria developed on this agar in 24 hours as on ordinary agar.

The following tables show the results obtained from the agglutination tests.

TABLE 1

TESTS OF RABBITS IMMUNIZED WITH STRAIN GROWN ON SUGAR-FREE AGAR

Medium	Rabbit	Control	1/50*	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar......	1	—	+++	+++	++	++	++	++	+
	2	—	+++	++	++	++	+	+	tr.
Blood agar...........	1	—	++	++	++	+	tr.	tr.	—
	2	—	++	+	+	+	tr.	—	—
Loeffler's serum......	1	—	+++	++	++	++	++	+	tr.
	2	—	+++	++	++	+	+	tr.	—
Dextrose..............	1	—	++	++	++	++	++	+	tr.
	2	—	+++	++	++	++	+	tr.	—
Lactose...............	1	—	++	++	++	++	+	+	tr.
	2	—	+++	++	++	++	+	tr.	—
Potato................	1	—	+++	++	++	++	++	+	tr.
	2	—	+++	++	++	+	+	—	—
Nonprotein...........	1	—	+++	+++	++	++	+	+	tr.
	2	—	+++	+++	++	++	+	tr.	—

* Explanation: +++ = complete agglutination; deposit and supernatant fluid clear. ++ = Marked agglutination; deposit but supernatant fluid not clear. + = Slight agglutination; flakes visible to the eye. tr. = Trace. — = No agglutination.

TABLE 2

TESTS OF RABBITS IMMUNIZED WITH STRAIN GROWN ON BLOOD AGAR

Medium	Rabbit	Control	1/50	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar......	1	—	++	++	++	++	+	tr.	tr.
	2	—	++	++	++	+	+	+	—
Blood agar...........	1	—	+++	++	++	++	++	++	tr.
	2	—	+++	++	++	++	++	+	tr.
Loeffler's serum......	1	—	++	++	++	+	tr.	—	—
	2	—	+++	++	++	++	+	+	tr.
Dextrose..............	1	—	++	++	++	++	++	tr.	tr.
	2	—	+++	++	++	++	+	+	tr.
Lactose...............	1	—	++	++	++	+	tr.	—	—
	2	—	+++	++	++	++	+	+	tr.
Potato................	1	—	++	++	++	+	tr.	—	—
	2	—	+++	++	++	++	+	+	tr.
Nonprotein...........	1	—	+++	++	++	++	+	tr.	—
	2	—	+++	++	++	+	+	tr.	tr.

TABLE 3

TESTS OF RABBITS IMMUNIZED WITH STRAIN GROWN ON LOEFFLER'S SERUM

Medium	Rabbit	Control	1/50	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar......	1	—	+++	++	++	++	+	+	+
	2	—	+++	++	++	+	+	+	tr.
Blood agar...........	1	—	+++	++	++	++	++	+	+
	2	—	+++	++	++	++	++	+	tr.
Loeffler's serum......	1	—	+++	+++	++	++	++	++	+
	2	—	+++	+++	++	++	++	+	+
Dextrose............	1	—	+++	++	++	++	++	+	+
	2	—	+++	++	++	++	+	+	+
Lactose...............	1	—	+++	++	++	++	++	+	+
	2	—	+++	++	++	++	+	+	tr.
Potato...............	1	—	++	++	++	++	++	+	tr.
	2	—	+++	++	++	++	++	+	—
Nonprotein...........	1	—	+++	++	++	++	+	+	tr.
	2	—	+++	++	++	++	++	+	tr.

TABLE 4

TESTS OF RABBITS IMMUNIZED WITH STRAIN GROWN ON DEXTROSE BROTH

Medium	Rabbit	Control	1/50	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar......	1	—	++	++	++	++	+	tr.	—
	2	—	+++	++	++	++	++	tr.	—
Blood agar...........	1	—	++	++	++	+	—	—	—
	2	—	++	++	++	+	+	tr.	—
Loeffler's serum......	1	—	+++	++	++	++	++	+	tr.
	2	—	+++	++	++	+	+	tr.	—
Dextrose..............	1	—	+++	+++	+++	++	++	+	+
	2	—	+++	++	++	++	++	++	+
Lactose..............	1	—	+++	+++	++	++	++	+	tr.
	2	—	+++	++	++	++	++	+	tr.
Potato...............	1	—	+++	++	++	++	++	+	tr.
	2	—	+++	++	++	++	+	+	tr.
Nonprotein...........	1	—	+++	++	++	++	+	+	tr.
	2	—	+++	++	++	+	+	+	tr.

TABLE 5

TESTS OF RABBITS IMMUNIZED WITH STRAIN GROWN ON LACTOSE BROTH

Medium	Rabbit	Control	1/50	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar	1	—	+++	++	++	++	++	+	—
	2	—	+++	++	++	++	+	+	tr.
Blood agar	1	—	++	++	++	+	+	tr.	—
	2	—	++	++	+	+	+	tr.	—
Loeffler's serum	1	—	+++	++	++	++	+	+	tr.
	2	—	+++	++	++	++	++	+	tr.
Dextrose	1	—	+++	++	++	++	++	+	+
	2	—	+++	++	++	++	++	++	+
Lactose	1	—	+++	+++	++	++	++	+	tr.
	2	—	+++	++	++	++	++	+	+
Potato	1	—	+++	++	++	++	++	+	tr.
	2	—	+++	++	++	++	++	+	tr.
Nonprotein	1	—	+++	++	++	++	+	+	—
	2	—	+++	++	++	++	+	+	tr.

TABLE 6

TEST OF RABBIT IMMUNIZED WITH THE STRAIN GROWN ON POTATO

	Control	1/50	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar	—	+++	++	++	++	++	+	—
Blood agar	—	+	+	+	+	tr.	—	—
Loeffler's serum	—	+++	++	++	++	++	++	+
Dextrose	—	+++	++	++	++	++	++	+
Lactose	—	+++	++	++	++	++	+	—
Potato	—	+++	++	++	++	++	+	tr.
Nonprotein	—	+++	++	++	++	+	+	+

TABLE 7

TEST OF RABBIT IMMUNIZED WITH THE STRAIN GROWN ON NONPROTEIN AGAR

	Control	1/50	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar	—	+++	++	++	++	++	+	tr.
Blood agar	—	++	++	+	+	tr.	—	—
Loeffler's serum	—	+++	++	++	++	++	+	tr.
Dextrose	—	++	++	++	++	++	+	+
Lactose	—	+++	+++	++	++	++	+	tr.
Potato	—	+++	++	++	++	++	+	tr.
Nonprotein	—	+++	+++	++	++	++	++	+

TABLE 8

TEST OF RABBIT IMMUNIZED WITH STRAIN GROWN ON PLAIN AGAR, 5 INTRAPERITONEAL INJECTIONS

	Control	1/50	1/250	1/500	1/1000	1/2000	1/5000	1/10000
Sugar-free agar	—	+++	++	++	++	++	++	+
Blood agar	—	++	++	+	+	tr.	tr.	tr.
Loeffler's serum	—	+++	++	++	++	++	++	+
Dextrose	—	+++	++	++	++	++	+	+
Lactose	—	+++	++	++	++	++	++	+
Potato	—	+++	++	++	++	++	++	+
Nonprotein	—	+++	+++	++	++	++	++	+

SUMMARY AND CONCLUSIONS

A typical paratyphoid B bacillus was transferred 44 times on each of the following mediums: Sugar-free agar; 10% rabbit blood agar; Loeffler's serum; 1% dextrose broth; 1% lactose broth; potato, and Jordan's nonprotein medium. Two rabbits were immunized with each strain produced on these mediums. The period of immunization was the same for all the rabbits. In both the injection of the rabbits and the agglutination tests the suspensions of the bacteria used were standardized (approximately 100,000,000 per c c). The following results were obtained:

Each strain, as a rule, agglutinated better with its homologous serum than with any heterologous serum. The serum produced by the potato strain, however, was an exception, since it agglutinated the organisms grown in dextrose broth and on Loeffler's serum better than its own strain.

The variations in agglutination were not marked, consisting merely of slight differences in the highest dilutions to which the strains are agglutinated.

The effect of growth on blood agar was most pronounced, for altho the blood agar strain by no means showed complete loss of agglutinability with heterologous serums, yet it was agglutinated to a far less degree by these serums than by its own, except in the case of the serum of the rabbits immunized with the Loeffler's serum strain. The blood agar strain did not, however, produce a serum of higher agglutinating titer for heterologous strains than did the other strains.

The fact that the strain grown on Loeffler's serum did not show a similar decrease in agglutinability might be explained either by the absence of red blood corpuscles or by the presence of the sugar in the medium.

The strains grown on dextrose and lactose broths produce serums with a relatively high titer for both homologous and heterologous strains of bacteria. But they did not show the marked increase in their agglutinogenic properties noted by Simonds[14] with the typhoid bacillus.

A slight increase in the amount of the agglutinogenic substance produced toward organisms cultivated on other carbohydrate-containing mediums was noted in the potato strain.

The nonprotein agar strain did not show a marked decrease in its agglutinative property such as was observed by Gloessner in his work with B. typhosus.

One rabbit immunized for a longer period of time than the others showed a marked increase in the titer of its serum. Possibly, some changes in agglutination attributed to the increase in the agglutinogenic property of the bacteria may be due to variations in the time element in immunizing the rabbits.

THE METHYL RED AND VOGES-PROSKAUER REACTIONS WITH SPECIAL REFERENCE TO ROUTINE WATER ANALYSIS

W. D. STOVALL AND M. STARR NICHOLS

From the Wisconsin State Laboratory of Hygiene, University of Wisconsin

Bacteriologic examination of water is an important part of the work of public health laboratories. It is essential that the method employed for these examinations be simple, easily applied to a large number of examinations, and that it distinguish factors of sanitary significance. This means that pathogenic bacteria must actually be isolated or that some condition, which is habitually associated with pathogenic bacteria be established. It must show actual danger to the health of those who drink the water, or a strong presumptive evidence of danger. The group of colon aerogenes organisms has been the indicator by which we have presumed the purity or pollution of water. If this group of organisms can be divided into at least two classes, one of which has a habitat in the intestinal tract of animals and the other in places not usually subject to animal pollution, then it is not proper to accept as an indicator of dangerous pollution all members of the colon aerogenes group.

In this laboratory we make a great many water analyses. We have used the methods of the "Standard Methods of Water Analysis of the American Public Health Association." A very high percentage of the water samples received have given positive tests for the colon group of bacteria. That is, lactose broth has been fermented with gas production, plates made from the gas tubes have shown acid colonies on litmus lactose agar, and acid colonies furnished from the plates have again produced gas from lactose broth. A good many of these samples did not show a high agar or gelatin count. We therefore for some time have been of the opinion that probably we were condemning water supplies as being polluted that should not have been. It is not to be inferred that we have applied the same standards to isolated wells and springs as to water supplies of villages and cities. It is hard to say just exactly why we have not, if the group of colon aerogenes organisms is accepted as an indicator of pollution. If, how-

Received for publication March 22, 1918.

ever, the two types of this group, methyl red positive and Voges-Proskauer positive organisms, represent, respectively, pollution of fecal and contamination of soil origin, then the permissible number of organisms present of the colon-aerogenes group will depend on the divisions into which they are classified. A much larger number of bacteria of soil origin can be permitted than those of fecal origin. Some of the recent work along this line gives proof of such a classification of the organisms of the colon aerogenes group of bacteria.

Rogers, Clark, and Lubs, and their associates have been able to show that bacteria belonging to the colon-aerogenes group can be classified with a few exceptions into two distinct classes, the high and low $CO_2:H_2$ ratio organisms.

Rogers, Clark and Davis[1] working with organisms obtained from milk say: "The carbon dioxid to hydrogen ratio which occurs with the greatest frequency is approximately 1:1. Plotted on the frequency basis this ratio stands apart from all higher cultures. All cultures giving the 1:1 ratio are distinguished from the high ratio cultures by the amount of gas formed under exact conditions. This was uniformly less than the amount produced under identical conditions by the high ratio cultures."

Rogers, Clark and Evans[2] in their two papers giving the results of their work with organisms isolated from cows' feces and grain give further proof of their classification of members of the colon-aerogenes group of bacteria and say: "The low ratio group evidently includes those members of the colon group usually designated as Bacillus coli communis and Bacillus coli communior, while in the high ratio group we may recognize Bacillus lactis aerogenes, Bacillus acidi lactici and possibly Bacillus coloacae."

Clark and Lubs[3] by the use of indicators have described a very simple test, which distinguishes between the high and low $CO_2:H_2$ ratio organisms. This test is applicable to routine water examinations.

Levine[4] was able to show a correlation between the methyl red and the Voges-Proskauer reactions. The organisms alkaline to methyl red gave a positive Voges-Proskauer reaction, and vice versa. He says that organisms which give a positive Voges-Proskauer test are seldom found in feces and that the Voges-Proskauer like the high ratio and alkalinity to methyl red is characteristic of nonfecal strains.

Procedure

During the spring, summer and fall we receive a large number of water samples collected from private and public supplies. These samples are collected in special containers prepared in the laboratory for this purpose. They are shipped in cases especially constructed for the preservation of the sample while en route. For our routine examinations we have used the methods prescribed by the Standard Methods of Water Analysis of the Am. Pub. Health Assn. with the exception of the amounts of water with which the fermentation tubes are inoculated. Five 10 c c portions from each sample of water was inoculated into each of 5 fermentation tubes, containing approximately 40 c c of a 1% lactose broth in place of the 10 c c, 1 c c and 0.1 c c portions of the

[1] Jour. Infect. Dis., 1914, 14, p. 411.
[2] Ibid., 1914, 15, p. 99.
[3] Ibid., 1915, 17, p. 160.
[4] Ibid., 1916, 18, p. 358.

Standard Methods. All organisms have been required to satisfy the completed test of the Standard Methods and to be gram-negative before accepted as a member of the colon group.

In the preparation of the mediums used in our routine water examination as in all other procedures, we have followed the Standard Methods for Water Analysis. The peptone, glucose, phosphate mediums of Clark and Lubs[3] has been used for the methyl red and Voges-Proskauer reactions. Test tubes were filled with this medium and after inoculation were incubated at 37 C. for 3 days. At the end of the incubation period the culture was divided approximately in half, one half being used for the methyl red test and the other for the Voges-Proskauer reaction. The potassium hydroxid used in the Voges-Proskauer test was a 10% solution of Mercks' "Highest Purity." The methyl red was prepared by dissolving 1 gm. of Kahlbaum's methyl red in 1,000 c c of 95% alcohol. It was found that 0.2 c c of this solution was turned a distinct red in 100 c c of a $\frac{n}{100,000}$ solution of H_2SO_4. The neutral control remained yellow. The results of these tests are compared on 200 organisms isolated from water as described above.

RESULTS

Tables 1, 2 and 3 have been arranged to show the number of cultures isolated from each water sample, the reactions of each culture to methyl red and Voges-Proskauer tests, when grown in the dipotassium phosphate medium of Clark and Lubs, the intensity of these reactions, and the gas produced in 24 and 48 hours in pure and mixed culture. The gas was measured by noting the displacement of the medium down the closed arm of the fermentation tube, and is expressed in per cent. We realize the inaccuracies of this rough estimation, but believe for our purpose it is significant. We have used the average gas production by members of a type to indicate the influences of certain factors, growth in mixed and pure culture and incubation temperature, on the growth of members of that type.

It will be seen from Tables 1 and 2 that there was 120 of the 200 organisms which gave a positive methyl red test and 38 a positive Voges-Proskauer reaction, when first isolated. These organisms gave an undoubted reaction when first isolated, and reacted in the same manner to the second test. Table 3 shows the organisms which have reacted irregularly either by a double test or because in the second test the reaction was different from that obtained in the first test. In the first test there were 29 organisms which either gave a slight reaction to the methyl red and a comparatively strong reaction to the Voges-Proskauer tests or vice versa or a reaction of equal intensity to both tests. Because of these irregular reacting organisms, we decided to test all organisms again for the Voges-Proskauer and methyl red reactions, and gas production in pure culture.

TABLE 1

METHYL RED POSITIVE ORGANISMS

Water Sample	Culture Number	Gas in Lactose Mixed 24 Hours	Gas in Lactose Mixed 48 Hours	Gas in Lactose Pure 24 Hours	Gas in Lactose Pure 48 Hours	Peptone Phosphate 1st Test MR	Peptone Phosphate 1st Test VP	Peptone Phosphate 2d Test MR	Peptone Phosphate 2d Test VP
QA	132	30	40	5	10	4+	0	3+	0
	133	40	50	30	35	4+	0	4+	0
	134	40	40	35	35	4+	0	3−	0
	136	25	30	25	30	4+	0	4+	0
XA	162	10	40	0	20	4+	0	4+	0
	163	10	50	15	20	4+	0	2+	0
	164	15	35	0	20	4+	0	4+	0
	165	5	35	5	15	4+	0	4+	0
PB	178	35	40	30	35	+	0	4+	0
	185	15	20	30	35	+	0	4+	0
	186	40	60	10	20	+	0	4+	0
	189	35	60	10	15	+	0	4+	0
AB	175	60	90	35	40	4+	0	3+	0
	176	35	60	25	35	4+	0	3+	0
	177	40	90	25	30	4+	0	4+	0
	179	45	60	25	30	4+	0	4+	0
X	65	5	35	10	15	+	0	3+	0
	66	15	25	5	10	+	0	3+	0
	69	10	30	5	25	+	0	4+	0
YA	166	5	10	35	35	4+	0	4+	0
	167	15	30	25	30	4+	0	4+	0
	168	15	20	35	40	4+	0	4+	0
D	8	20	40	20	30	+	0	4+	0
	9	10	30	15	30	+	0	4+	0
E	11	10	15	5	15	+	0	4+	0
	12	25	30	5	10	+	0	4+	0
F	14	30	40	25	25	+	0	4+	0
	15	40	50	35	35	+	0	2+	0
G	16	5	5	5	5	+	0	4+	0
	17	10	10	5	10	+	0	4+	0
H	19	5	15	0	10	+	0	4+	0
	20	5	15	5	5	+	0	4+	0
J	25	40	40	25	35	+	0	3+	0
	26	30	20	30	35	+	0	4+	0
P	42	0	10	15	30	+	0	4+	0
	43	0	10	30	40	+	0	4+	0
LB	117	35	40	60	60	+	0	3+	0
	118	40	50	15	30	+	0	3+	0
PA	129	10	60	5	5	1+	0	2+	0
	130	5	60	5	5	4+	0	3+	0
UA	138	5	20	35	35	4+	0	3+	0
	139	0	5	0	0	4+	0	4+	0
MB	141	25	35	40	40	+	0	4+	0
	146	40	40	60	80	+	0	3−	0
A	1	10	20	30	30	+	0	4+	0
C	7	10	35	30	30	+	0	3+	0
K	27	15	20	15	20	+	0	4+	0
M	47	0	15	20	30	+	0	4+	0
W	63	5	50	20	30	+	0	4+	0
Z	74	30	30	20	20	+	0	4+	0
DA	86	0	40	20	20	+	0	4+	0
NA	102	10	10	20	25	+	0	3+	0
GA	103	10	20	15	20	+	0	4+	0
IB	106	5	30	25	30	4+	0	4+	0
MA	119	0	30	0	15	3+	0	3+	0
SA	150	0	40	5	10	4+	0	4+	0
EB	195	10	40	30	35	4+	0	4+	0
FB	196	25	40	40	40	4+	0	4+	0
N	36	20	20	20	25	+	0	4+	0
	37	30	40	20	25	+	0	4+	0
	38	10	15	25	30	+	0	4+	0
	39	30	30	30	30	+	0	4+	0
	40	20	20	30	40	+	0	4+	0
T	46	35	40	35	35	+	0	4+	0
	57	25	25	20	30	+	0	4+	0
	58	15	15	20	25	+	0	4+	0
	59	20	20	20	30	+	0	4+	0
	60	20	25	15	25	+	0	4+	0
ZA	170	40	40	35	40	3+	0	3+	0
	171	40	40	30	40	3+	0	4+	0
	172	40	40	40	40	3+	0	2+	0
	173	50	60	40	40	3+	0	2+	0
	174	40	60	40	40	2+	0	3+	0
BB	180	30	30	20	40	4+	0	4+	0
	181	30	35	25	40	4+	0	4+	0
	182	30	35	40	40	4+	0	4+	0
	183	40	40	40	40	4+	0	4+	0
	184	40	40	20	35	4+	0	4+	0
HB	6	10	15	30	40	+	0	4+	0
	121	20	35	20	30	3+	0	4+	0
	123	30	35	10	15	3+	0	4+	0
	135	25	30	40	40	4+	0	4+	0
	137	30	40	25	30	4+	0	3+	0
NB	147	35	40	50	60	+	0	4+	0
	148	20	30	50	50	+	0	4+	0
	149	30	40	50	50	+	0	4+	0
	151	25	35	70	70	+	0	4+	0
	155	25	35	50	50	+	0	4+	0
B	2	5	30	0	10	+	0	4+	0
	3	10	25	5	5	+	0	4+	0
	4	10	30	15	30	+	0	4+	0
	5	15	30	5	5	+	0	4+	0
DB	13	0	30	30	35	+	0	3+	0
	188	10	40	40	40	2+	0	4+	0
	190	35	60	10	15	+	0	4+	0
	191	25	35	25	30	4+	0	4+	0
GB	20	5	15	5	5	+	0	4+	0
	21	0	15	10	20	+	0	4+	0
	22	0	5	0	0	+	0	3+	0
	198	60	60	35	35	4+	0	4+	0
KB	23	50	50	40	40	+	0	4+	0
	87	50	50	40	40	+	0	4+	0
	88	40	50	15	20	+	0	4+	0
	96	40	60	60	60	+	0	4+	0
L	31	5	5	30	30	+	0	4+	0
	32	5	30	25	30	+	0	4+	0
	33	10	20	30	35	+	0	3+	0
	34	10	10	25	30	+	0	3+	0
S	51	25	25	15	20	+	0	3+	0
	53	40	40	30	35	+	0	4+	0
	54	20	20	35	40	+	0	4+	0
	55	20	25	15	30	+	0	4+	0
CA	89	10	40	5	10	+	0	4+	0
	90	15	25	20	25	+	0	4+	0
	91	15	25	25	40	+	0	4+	0
	92	5	20	5	25	+	0	4+	0
FA	97	0	40	20	20	4+	0	4+	0
	98	20	40	25	25	+	0	4+	0
	99	0	20	30	30	4+	0	4+	0
	100	0	30	10	20	4+	0	4+	0

A study of Tables 1 and 2 with reference to the gas produced after 24 hours in the mixed and pure cultures shows that in 29% of the methyl red positive organisms there was an increase of 20% or more in the gas formation in the second 24 hours' incubation in the mixed

TABLE 2

VOGES PROSKAUER POSITIVE ORGANISMS

Water Sample	Culture Number	Gas in Lactose				Peptone Phosphate				Water Sample	Culture Number	Gas in Lactose				Peptone Phosphate			
		Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	1st Test MR	1st Test VP	2d Test MR	2d Test VP			Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	1st Test MR	1st Test VP	2d Test MR	2d Test VP
AA	77	30	80	60	80	0	+	0	3+	EB	192	35	60	60	90	0	1+	0	2+
	78	5	50	20	60	0	+	0	4+		193	5	50	35	70	0	2+	0	1+
	79	40	90	60	80	0	+	0	2+		194	10	40	0	30	0	3+	0	2+
	80	30	50	40	60	0	+	0	1+	EA	29	20	70	35	80	0	+	0	2+
	81	10	40	20	60	0	+	0	3+		30	0	80	30	50	0	4+	0	3+
Z	72	50	50	80	90	0	+	0	3+	QB	46	60	90	50	60	0	4+	0	4+
	73	40	40	5	10	0	+	0	1+		48	10	80	40	40	0	4+	0	4+
	75	50	70	60	90	0	+	0	4+	R	49	10	10	35	60	0	+	0	2+
	76	40	65	60	90	0	+	0	3+		50	15	20	10	30	0	+	0	1+
OB	157	40	50	70	90	0	+	0	3+	Q	28	0	5	35	60	0	+	0	1+
	159	30	50	90	90	0	2+	0	3+	BA	35	10	50	40	70	0	+	0	3+
	160	25	40	90	90	0	+	0	4+	P	41	0	15	15	30	0	+	0	2+
	161	25	35	50	90	0	+	0	1+	S	52	25	35	35	40	0	+	0	2+
W	61	5	20	15	30	0	+	0	3+	X	67	15	15	20	40	0	+	0	2+
	62	0	40	5	10	0	+	0	1+	V	71	0	10	0	0	0	+	0	4+
	64	5	30	10	30	0	+	0	2+	Y	82	0	40	10	20	0	+	0	3+
HA	108	0	10	10	40	0	3+	0	3+	OA	124	80	90	20	30	0	3+	0	4+
	109	0	25	10	30	0	2+	0	2+	PA	131	5	50	5	35	0	3+	0	3+
	110	0	10	10	30	0	2+	0	4+	WA	158	5	5	5	35	0	1+	0	2+

TABLE 3

IRREGULAR REACTING ORGANISMS

Water Sample	Culture Number	Gas in Lactose				Peptone Phosphate				Water Sample	Culture Number	Gas in Lactose				Peptone Phosphate			
		Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	1st Test MR	1st Test VP	2d Test MR	2d Test VP			Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	1st Test MR	1st Test VP	2d Test MR	2d Test VP
JA	112	0	30	5	15	2+	2+	2+	3+	OA	125	60	70	40	60	1+	1+	3+	1+
	113	0	20	5	15	0	2+	1+	3+		126	10	60	10	20	1+	1+	2+	3+
	114	0	20	15	20	2+	1+	2+	2+	PA	127	60	70	5	20	1+	2+	2+	0
	115	0	20	5	30	2+	1+	2+	1+		128	60	70	0	5	3+	2+	2+	4+
	116	0	15	10	40	2+	1+	2+	1+	U	70	0	10	5	20	+	+	3+	1+
TA	142	0	30	0	10	0	3+	1+	3+	Y	83	0	20	5	15	+	+	3+	3+
	143	0	30	5	40	1+	2+	2+	3+	KA	94	0	30	40	40	+	+	2+	1+
	144	0	25	5	30	2+	2+	2+	2+	MA	120	0	40	5	40	2+	1+	2+	4+
	145	5	10	5	50	1+	3+	1+	2+	YA	169	15	25	10	35	1+	1+	2+	1+
VA	152	5	35	10	40	0	2+	2+	3+	E	10	0	5	20	30	+	+	0	1+
	153	10	45	5	50	0	4+	3+	3+	G	18	0	15	40	60	+	+	0	1+
	154	10	20	0	30	0	1+	2+	2+	I	24	0	5	15	30	+	+	0	1+
	156	5	15	0	30	1+	3+	2+	3+	UA	140	0	10	5	35	1+	3+	0	2+
GA	104	0	20	5	60	0	2+	1+	3+	LA	95	0	30	20	40	+	+	0	2+
	105	0	25	5	40	1+	1+	1+	4+	CB	187	0	30	0	0	1+	1+	3+	0
	107	0	20	5	15	1+	2+	3+	0	FB	197	5	40	35	40	2+	1+	4+	0
O	44	0	10	10	30	+	+	3+	1+	JB	111	40	60	0	0	0	2+	2+	3+
	45	0	10	15	30	+	+	2+	0	X	68	5	15	0	0	0	+	4+	0
IA	84	0	10	5	10	0	+	1+	3+	CA	93	5	30	0	0	0	0	1+	0
	85	0	35	5	10	+	+	1+	4+	GB	199	40	50	25	90	0	3+	0	0
NA	101	10	30	0	0	+	2+	4+	0										
	122	0	10	40	50	2+	1+	2+	1+										

cultures, and that in the pure cultures only 4% have shown as much as 20% increase in gas after the first 24 hours.

In the group of Voges-Proskauer positive organisms a little more than 57% had an increase of 20% or more of gas in the second 24 hours, in the mixed culture, and 66% shows a similar increase when grown in pure culture.

In pointing out in the foregoing the effects of growth in a mixed culture on the growth of the different groups of organisms as indicated by gas formation, individual cultures, without reference to the samples of water from which they came, have been used. Tables 1, 2 and 3 indicate that some samples of water yielded organisms belonging to the same group from all five of the portions planted, while others yielded organisms of two or more types. This is conveniently shown in the summary which follows.

From 28 waters all cultures isolated were M. R. +.
From 10 waters all cultures isolated were V. P. +.
From 19 waters some cultures isolated were M. R. + and some V. P. + and some irregular.
From 11 waters all cultures gave an irregular reaction.

It is therefore reasonable to conclude that in certain (28) samples of water only colon organisms of the methyl red group were present, and in certain other samples (10) only colon organisms of the Voges-Proskauer group were present. If, now, we consider only cultures which came from water samples from which all cultures isolated had a like reaction, we find in the average gas production in the first 24 hours in mixed and pure culture quite a difference. It is seen then that there are 10 water samples all cultures from which were positive to the Voges-Proskauer reaction. These cultures gave an average gas formation of 17% in the first 24 hours in the mixed culture, and 37% in the same period when growing in pure culture. The methyl red cultures selected and compared in the same manner show no difference in the gas formation in the mixed and pure cultures. These results are shown in Table 4.

At the same time the fermentation tubes were inoculated with portions from the water samples for examination, agar and gelatin plates were also made. We thought that possibly there might be some correlation between the agar and gelatin counts and the reaction of the organisms isolated. Table 5 shows the results of agar and gelatin counts in water samples from which all cultures from the sample reacted alike.

We realize that there are too few water samples reported in this series from which to draw any absolute conclusion. It is seen, however, that 50% of the samples giving only Voges-Proskauer positive organisms give a high gelatin count and 30% a high agar count, and 30% high in both. Those samples from which organisms of the methyl red group only were obtained show a high gelatin count in 48+%, high agar count in 44+%, and high in both in 40+%. It must be borne in mind that some of these water samples were collected

TABLE 4

SHOWING THE AVERAGE GAS FORMATION IN THE FIRST 24 HOURS' INCUBATION OF THE METHYL RED POSITIVE AND VOGES-PROSKAUER POSITIVE ORGANISMS IN MIXED AND PURE CULTURE

	Water Samples	Culture Isolated	Gas Percent Culture	
			Mixed	Pure
V. P. +	10	22	17	37
M. R. +	28	85	25	25

from farm wells, and that in most cases they were exposed to gross pollution, which, of course, included many soil bacteria as well as bacteria of fecal origin.

In the first set of tests we found 29 organisms which gave an irregular reaction. We at first decided to do a duplicate test on these and then later decided to do a duplicate test on all of the cultures. The first set of tests therefore represent the reactions of the organisms

TABLE 5

SHOWING THE RELATION OF THE AGAR AND GELATIN COUNTS OF WATERS YIELDING ONLY ONE TYPE OF ORGANISMS

Organism Type	Number Waters	High Gelatin Count (1,000 or Above per C C)	High Agar Count (100 or Above per C C)	High in Both	Low in Both (Below 1,000 on Gelatin; Below 100 on Agar per C C)
V. P. +	10	5	3	3	5
M. R. +	28	13	12	11	14

when they were first isolated, and the second set the reactions about 6 months later. We soon learned that some organisms would apparently give a double positive reaction, that is, both a Voges-Proskauer and a methyl red. In some cases one would be much stronger than the other so that if we had classified them according to the intensity of the color reaction many would have been included in our groups of methyl red and Voges-Proskauer positive organisms which have not been. Some of the reactions gave a double positive reaction of

equal intensity, altho such reactions were usually weak in both cases, as is shown in Table 3. Because of this variation in intensity we marked the reactions as 1+, 2+, 3+, and 4+, according to whether the color was slightly red, red, a deep red, or a very deep red, and similarly with the pink in the Voges-Proskauer reaction.

Table 3 shows the results of the test of the irregular organisms. We have placed in this class every organism which has not reacted positive to either one test or the other and so reacted throughout both tests. We therefore have in this group all organisms which gave a double reaction regardless of the intensity of the reaction, and all which in any way had changed reaction in the second test. This makes the group of irregular reactors seem rather high. We are sure, however, that we have no organisms in either of the positive groups which do not belong there.

Table 3 is arranged to show all of the reactions of these organisms. It will be seen that 20 gave a double positive reaction in both tests, that 5 changed from a double positive to a Voges-Proskauer positive, that 6 changed from a double positive to a methyl red positive, that 8 changed from a Voges-Proskauer positive to a double reaction, and that Nos. 68, 93, and 199 were irregular to one or more of the reactions. Of the 25 waters, which furnished the 42 irregular cultures, 14 furnished one or more methyl red positive or Voges-Proskauer positive, or Voges-Proskauer and methyl red positive organisms along with the irregular reactors. Eleven furnished only irregular organisms. One water sample furnished an irregular reactor from each of the 5 fermentation tubes inoculated and 2 samples four each from the 5 tubes planted.

We hope to report soon on the classification of these irregular reactors by accurate gas analysis.

DISCUSSION

The influence of growth in mixed cultures on the isolation from water of organisms of the methyl red and Voges-Proskauer positive types in the colon group is of particular interest in the routine examination of water. It has been shown by Clark and Roger and others that in pure culture under anaerobic conditions the methyl red positive organisms produce a smaller amount of gas than the Voges-Proskauer, and further that the methyl red positive type reach their limit of H-ion concentration and stop growing, while the Voges-Proskauer type may form acid to a certain H-ion concentration, and

then certain other reactions intervene to reduce the H-ion concentration formed and this process starts over.

In the fermentation tube which has growing in it many types of bacteria, which may be found in almost any sample of water, conditions are entirely different and the reactions therefore must be different. In those tubes which have been inoculated with portions from water samples, both methyl red positive, and Voges-Proskauer positive organisms, not to mention the variety of other bacteria, may be growing. A study of the gas produced in Tables 1 and 2 we think throws some light on how these two types react in mixed and pure culture. We have not compared the volume of gas produced by each individual culture because we were aware of the errors into which we would fall. We have, however, used the average gas production of the whole group for the periods which we wished to compare, and believe that the results indicate what has actually happened.

We find that by comparing the gas produced in the first 24 hours in fermentation tubes from which methyl red positive organisms were isolated with the average amount of gas produced in the second 24 hours' incubation, that 29% show an increase of 20% or more in gas produced. A similar comparison made after the culture was isolated and growing in pure culture shows that only 4% of the cultures show such an increase. However, the average amount of gas formed (25%) in tubes which were inoculated from samples of water from which only methyl red positive organisms were isolated during the first 24 hours' incubation was exactly the same as that formed by these organisms when growing in pure culture. We believe, therefore, that other reactions brought about by other organisms reduced or partially reduced the H-ion concentration until the organisms isolated established its predominance in the second 24 hour period of incubation. It appears, therefore, that the methyl red positive organisms growing in pure culture may not establish their predominance until after 24 hours' incubation at 37 C. It appears that when Endo plates made from tubes showing gas in the first 24 hours fail to show characteristic colon colonies, plates made at the end of 48 hours may show such colonies in numbers.

In the case of the Voges-Proskauer positive types found in our group a marked difference in the average amount of gas produced in the pure and mixed culture, and in the average increase in the second 24 hours was found. In this group 57% growing in the mixed

culture, and 66% growing in pure culture showed 20% or more increase in gas produced in the second 24 hours.

When we examine the average gas production in tubes inoculated from water samples from which only Voges-Proskauer organisms were isolated during the first 24 hours in the mixed and pure culture, a marked difference is noted. The average gas production in the mixed culture was only 17% while in the pure culture it was 37%. When we put these figures together we find that a considerably smaller number gave as much as a 20% increase in gas formation during the second 24 hours in the mixed culture, and also that the average gas production was considerably less in the first 24 hours. This indicates that the organisms of this type have been inhibited to a considerable degree from some cause. We believe that it may be the combination of the effect of growth in the same tube with many other organisms and the temperature, 37 C., of incubation. It is probable that the Voges-Proskauer positive class of the colon aerogenes group being of a nonfecal type do not find so high a temperature advantageous for their growth. On the other hand, the methyl red class being a fecal type would find such a temperature highly satisfactory for their development.

It is probable, therefore, that under the condition in which our routine water analysis is done we are favoring the growth of methyl red positive class of organisms, and inhibiting the growth of the Voges-Proskauer. It appears that the methyl red positive division is not inhibited to any degree by growing in mixed culture at 37 C., while the Voges-Proskauer are. This may be interpreted to mean that the methyl red positive division being direct pollution from the intestinal tracts of animals finds this temperature most favorable, while the other division, commonly soil bacteria, finds it unfavorable.

SUMMARY

In the routine analysis of 68 water samples from private and public supplies received from many sections of the state we have isolated 200 cultures which fulfilled the requirements of the completed test for members of the colon group of organisms of the Standard Methods of Water Analysis. We found that 120 of these reacted acid to methyl red, 38 gave a Voges-Proskauer positive reaction, and that 42 gave irregular reactions.

The intensity of these reactions varied considerably. In instances in which the same organism apparently gave both reactions positive it

was not always possible by the intensity of the reaction to say in what division it should be classed. In other instances, if the more intense reaction had been accepted as indicating the division to which the organism belonged, our group of irregular reacting organisms would have been much smaller.

It seems probable that the class of methyl red positive organisms is not interfered with in its development when growing with other bacteria found in water incubated at 37 C. as are the Voges-Proskauer. It is probable that in the same culture the methyl red positive will overgrow the Voges-Proskauer positive organisms. It seems advisable therefore whenever these reactions are used in water analysis to make Endo or litmus-lactose agar streak plates from the fermentation tubes at the end of 24 and 48 hours.

We are not able in the small number of water samples here reported to find any correlation between the agar and gelatin count and the type of organisms of the colon group isolated from the samples of water.

A RESPIRATORY STIMULANT AND TOXIC SUBSTANCE EXTRACTABLE FROM LUNG TISSUE

William B. Wherry and D. M. Ervin

From the Laboratories of Bacteriology and Pathology, University of Cincinnati

The interesting method we shall describe of producing accelerated respiration or death was discovered accidentally in an experiment with tuberculous tissue from a rabbit's lung. A piece of the tissue, 6 by 8 mm., filled with tubercles, was ground up with sand, suspended in 8 c c of 0.9% NaCl solution, centrifuged to remove coarse particles, and 1 c c of the supernatant fluid injected intravenously into another rabbit, which immediately fell on its side with marked inspiratory dyspnea, followed by convulsions and opisthotonos, and died a few seconds later.

We shall not detail the earlier experiments during which we thought we might be dealing with a toxin of the tubercle bacillus. Control experiments soon showed that tuberculosis played no part in the phenomenon.

We soon found that normal rabbit lung extract was not only fatal to rabbits, but also to guinea-pigs, when administered intravenously, though not to the same degree. On the other hand, guinea-pig lung extract is more fatal to guinea-pigs than to rabbits. It was only after a number of injections that we standardized our dose. This was done by weighing a piece of lung of about the same size as we had used before.

The Crude Lung Extract.—Lung tissue, 0.6 gm., was ground to a fine paste with sand in a mortar, and the grinding continued while 6 c c of 0.9% NaCl solution was added slowly. The toxicity of the extract was not affected appreciably by the time devoted to grinding, e. g., 3 or 10 minutes. The extract was then centrifuged at 1,200-1,500 revolutions a minute and the supernatant fluid pipetted off from below the slight scum which rises to the surface. It did not seem to make much difference whether the centrifugation was carried out only for a few minutes or for an hour or two at the speed mentioned. One c c of the crude extract does not really contain 0.1 gm. of lung tissue for most of the tissue remains either in the mortar or in the

Received for publication March 26, 1918.

centrifuge tube. Such an extract is deeply tinged with hemoglobin and may be opaque or translucent according to the centrifugation it has been subjected to. The fatal intravenous dose of this rabbit lung extract for rabbits varying from 800-2,000 gm. in weight, is 0.3 c c. This and larger doses kill in a few seconds; 0.1 c c fails to kill but markedly accelerates the respiration.

The guinea-pig lung extracts were made in the same way.

Symptoms.—If a rabbit be placed on the floor immediately after inoculation with 0.3 c c into an ear vein it may or may not lope off for a few steps. In a few seconds it becomes perfectly still, draws itself together, its respiration is accelerated; it sways a little from side to side; its respirations cease, and it falls on its side extended at full length. Then it goes into convulsions which may be violent or consist of a few running motions with all the legs, the head is retracted in extreme opisthotonos; the mouth opens; occasionally it cries out; one or more deep inspirations is followed by cessation of respiration. At this time the pupils are widely dilated and the superficial muscles of the body sometimes undergo rapidly repeated contractions, giving the appearance of violent shivering.

When dissected immediately after death, there is little abnormal found. The heart still beats after respiration ceases. There is no particular congestion of any of the organs. We have thought that the brain was abnormally pale. The lungs collapse on opening the thorax. The coagulability of the blood is delayed — altho we have not made a special study of this point, the delay is sometimes considerable, as shown in Rabbit 25. Intestinal peristalsis continues for some time as usual.

In the case of young rabbits which have received one or two sublethal doses we have twice observed a paralysis of the hind legs developing 3 or 4 days after inoculation (Rabbits 9 and 16). Rabbit 16 showed a noncoagulated hemorrhage into the cord at the level of the 5th-6th rib with complete softening of the cord. No bacteria were found in smears.

A sublethal dose in young as well as old rabbits accelerates the respiration at once to 30-40 per 10 seconds, while the normal resting rate is 12-16. There is a transitory but apparent weakness of the hind legs.

The following illustrates the results of inoculation of standard crude extract.

Rabbits Injected with Extract of Rabbit Lung.—Rabbit 6, 1,960 gm., 1 c c of clear extract centrifuged at 1,500 revolutions for 1 hour; dead in 2 minutes.

Rabbit 8, 1,870 gm., 1 c c in ear vein; dead in 30 seconds.

Rabbits Injected with Guinea-Pig Lung.—Rabbit 9, 600 gm., 0.5 c c; slight dyspnea only. Had survived Berkefeld filtrate of rabbit lung. Developed paralysis of both hind legs next day.

Guinea-Pigs Injected with Guinea-Pig Lung Extract.—We omit those in which we believe we failed to enter the vein in the foot. Guinea-pig 1, 250 gm., 0.5 c c; scratched nose. Few minutes later 1 c c; dyspnea, paralysis, no convulsions; dead in 15 minutes.

Guinea-pig 2, 320 gm., less than 0.5 c c; collapse, respirations 25 per 10 seconds, comatose on side, gasping; recovered 25 minutes later.

Guinea-pig 7, 250 gm., injected with 1 c c; dyspnea, convulsions in 1.5 minutes; dead in 6 minutes.

Guinea-pig 10, 250 gm., 1 c c; fell over in 1 minute, respirations 25 per 10 seconds, in 4, gasping in 9, and dead in 10 minutes.

Guinea-pig 11, 250 gm., 1 c c; dyspnea in 2 minutes; recovered.

A guinea-pig, which had been inoculated about a year previously from a guinea-pig dying of pernicious anemia, died of pneumonic consolidation of the lungs. No bacteria were found microscopically nor in cultures. Standard extract was made from this pneumonic lung and used as follows:

Guinea-pig 13, 300 gm., 0.5 c c; fell over in dyspnea in 20 seconds, rapid running motions with legs, occasional gasp, and death in 5 minutes.

Guinea-pig 14, 250 gm., 0.3 c c; dyspnea in 30 seconds, lying on side and passive 17 minutes later; recovered.

Guinea-pig 15, 300 gm., 0.4 c c; behaved like 13; died in 10 minutes.

Guinea-Pigs Injected with Rabbit Lung Extract.—Guinea-pig 3, 300 gm., 0.5 c c into tissues of foot and then 0.75 c c intravenously; weakness and dyspnea in 1 minute. Marked inspiratory dyspnea, and death in 3 minutes.

Guinea-pig 5, 800 gm., 3.5 c c intracardially; dyspnea and slight weakness only; recovered.

Negative Experiments with Similar Extracts of Liver, Kidney, Ileum and Spleen.—Rabbit liver extract made up like the lung extract, but an opaque, murky red fluid. Injected Rabbit 11, 1,230 gm., with 1 c c intravenously; no symptoms. This rabbit had been injected the day before with 1 c c of rabbit plasma which had been in contact with washed lung tissue sediment for 30 minutes and had shown no symptoms. The day after the liver extract inoculation it received 1 c c of lung extract diluted with 9 c c NaCl solution and died in 30 seconds.

Rabbit kidney extract in the form of a murky red fluid was injected into a vein of Rabbit j, 800 gm., in 0.5 c c dose without symptoms. Two days later it was injected with lung extract and died at once. Again a kidney extract was made up by weight and 10 c c of the extract in the form of a murky red fluid was inoculated intravenously into Rabbit 13, 2,030 gm.; no symptoms.

A few minutes later, Rabbit 13 was inoculated with a similar extract of the lower ileum of a rabbit; no symptoms. One hour after the last injection, Rabbit 13 received intravenously 1 c c of standard lung extract diluted to 10 c c with 0.9% NaCl solution; no symptoms at once, in 2 minutes dyspnea; in 3 minutes respirations were 38 per 10 seconds, in 8 minutes 41, in 12 minutes 44, and soon dropped to 20. Survived.

Whole rabbit spleen ground up with 10 c c salt solution; 1 c c intravenously into Rabbit 19, 1,350 gm.; no symptoms.

Experiments Showing that the Lethal Agent Does Not Pass the Berkefeld Candle N, and Is Removed by Animal Charcoal.—The standard rabbit lung extract was centrifuged at 1,200-1,500 revolutions until clear. Some of this was filtered through a thoroughly washed Berkefeld candle N which was known to be proof to B. prodigiosus. The filtrate was perfectly clear but red with hemoglobin.

Rabbit 7, 1,666 gm., injected intravenously with 1 c c; no symptoms. Kept under observation for a week when it was used as control (33) when it succumbed at once to the unfiltered extract.

Rabbit 9, 600 gm., injected with 2 c c of the filtrate; no symptoms. On the 4th day thereafter it developed paralysis of both hind legs.

Control Rabbit 8, 1,870 gm., injected with 1 c c of the unfiltered extract; dead in 30 seconds.

A 2,000 gm. rabbit was exsanguinated and the lungs washed out through the right ventricle with 0.9% salt solution. The very pale lungs were cut to pieces and these kneaded out in salt solution, dried with filter paper and the pale washed substance ground up to make standard extract which was centrifuged. The supernatant fluid was clear and straw colored.

Rabbit 27, 870 gm., injected intravenously with 0.3 c c of this extract; convulsions in 20 seconds, and dead in 45 seconds.

Rabbit 28, 890 gm.; the extract was passed through a Berkefeld N at 600 mm. pressure; filtered very slowly; injected 0.3 c c intravenously; no symptoms.

Rabbit 29, 830 gm.: the deposit on the outer surface of the filter used in the previous experiment was washed off with 3 c c of salt solution; a cloudy fluid resembling ovomucoid was obtained; injected 0.3 c c intravenously; dead in one minute; slight convulsions.

Rabbit 30, 650 gm.; the washings, used with Rabbit 29, were diluted to 4 c c and centrifuged at 1,200-1,500 revolutions for 20 minutes. The supernatant fluid was a perfectly clear, slightly opalescent fluid; injected 0.3 c c intravenously; no symptoms.

Rabbit 32, 1,200 gm., injected as Rabbit 30, but with 1 c c; convulsions in 80 seconds, and dead in 110 seconds.

Rabbit 31, 800 gm.; the unfiltered portion of the extract poured off from the Berkefeld filter, was used; injected 0.3 c c; convulsions and death in 30 seconds.

Rabbit 33, 1,660 gm., received 0.5 c c of standard lung extract intravenously; dead in 1.5 minutes.

The same lung extract was filtered through a layer of animal charcoal on hard filter paper. The filtrate looked like a solution of soap in water. Rabbit 34, 1,350 gm., received 0.5 c c intravenously. No symptoms. The filtrate was then centrifuged at 1,200-1,500 revolutions for 25 minutes; there was a moderate amount of sediment and opalescent fluid above. The sediment was mixed with 0.5 c c salt solution and injected intravenously in Rabbit 35, 1,300 gm.; marked inspiratory dyspnea, paralysis, nystagmus and convulsions with cry; dead in one minute.

The suspension of sediment which killed Rabbit 35 was examined and showed numerous flocculent masses varying from 2-12 microns in diameter and apparently made up of smaller visible particles. In addition there were quite a number of bacteria which apparently came from the animal charcoal. The supernatant fluid showed the same flocculi but smaller in size.

Standard extract of rabbit lung was made by grinding with sand and then with animal charcoal, which was added before the salt solution; the charcoal was centrifuged out; supernatant fluid clear like water; injected 1,200 gm. rabbit intravenously with 7 c c; no symptoms.

The Effect of Heat.—The crude rabbit lung extract was heated at 57 C. for 20 minutes; injected 2,000 gm. rabbit intravenously with 1 c c; died at once. Extract was boiled for one minute; coagulum centrifuged out at low speed; 2 c c of the slightly opalescent supernatant fluid was injected intravenously into a 800 gm. rabbit; in one minute it sneezed and respirations became accelerated. No other symptoms.

Tolerance Produced by Rapidly Repeated Inoculations or by Dilute Extracts.—1. Rabbit j, 800 gm., received 0.5 c c of a rabbit kidney extract intravenously without symptoms. Then it received rabbit lung extract intravenously as follows: 1/6 c c at 1-2 minute intervals until it had received 1 c c; no symptoms. A few minutes later, 0.5 c c intravenously; no symptoms. But this tolerance did not last, for 24 hours later when it received 1 c c of standard extract intravenously it died at once.

2. Rabbit h, 2,000 gm; rabbit lung tissue sediment was washed 3 times with salt solution; third supernatant fluid was injected as follows: 1 c c, 1.5 c c, 2 c c at minute intervals. No symptoms. Four days later it was inoculated intravenously with 1 c c of standard rabbit lung extract; it did not show symptoms until one minute when its respirations were accelerated; in 2 minutes it could barely stand up; it was paralyzed in 5, went into convulsions in 6, and died in 7 minutes.

Control Rabbit 3, 850 gm., received 0.5 c c of the same lung extract; dead in one minute without convulsions.

3. Standard rabbit lung extract was centrifuged at 1,200-1,500 revolutions for one hour. The supernatant fluid, clear and translucent, was pipetted off = A. The sediment was mixed with 4 c c of 0.9% NaCl solution and allowed to stand at room temperature for one hour; the supernatant fluid on this = B.

Rabbit 5, 1,960 gm., received intravenously 1 c c of B; no symptoms. Then 2 c c of B; no symptoms. Then 2 c c of A; no symptoms. The last inoculation would have killed a normal rabbit, as shown by Rabbit 6.

Control Rabbit 6, 1,960 gm., received intravenously 1 c c of A; dead in 2 minutes.

A lung sediment extract like that which gave protection to Rabbit 5 was centrifuged and the clear supernatant fluid tested for its protective action.

Rabbit 10, 1,500 gm., received 1 c c intravenously; no symptoms. Then it received 1 c c of standard lung extract; died in one minute.

Apparently something remained suspended in Extract B which stood for one hour that was capable of giving protection, but this was removed by centrifuging at high speed. This requires further confirmation.

Partial Protection Produced by Blood of a Shocked Rabbit.—Rabbit 25, 1,930 gm., received 0.3 c c standard rabbit lung extract. Dead in 2 minutes. Heart still beating 10 minutes later and blood not coagulated. The blood was allowed to flow into the thoracic cavity and 10 c c was injected into the ear vein of Rabbit 26, 1,660 gm. No reaction. About 10 minutes later injected 0.3 c c standard lung extract. No symptoms for 5 minutes, but it fell over in 6 and died in 9 minutes.

Incubation of Lung Extract with Homologous Corpuscles Does Not Increase Its Toxicity.—Made up standard lung extract from rabbit lung kept for 19 hours on ice = A.

Incubated some of A in water bath at 40 C. for 15 minutees = B.

Washed 1 c c rabbit corpuscles two times with salt solution; suspended in 3 c c of A and heated as B = C.

Rabbit 20, 1,685 gm., received 0.5 c c of B; convulsions in 20 seconds, down in 30, respirations 36 per 10 seconds; fine tremor over body in one minute; dead in 2 minutes.

Rabbit 21, 1,715 gm., received intravenously 0.3 c c of B; convulsions in 30, dead in 40 seconds.

Rabbit 22, 1,670 gm., received intravenously 0.1 c c of B; acceleration of respirations in one minute to 39 per 10 seconds. No other symptoms.

Rabbit 24, 1,880 gm., received intravenously 0.1 c c of C. No symptoms.

The Lethal Agent Is Not Removed by Ether.—Took 4 c c of standard rabbit lung extract and shook thoroughly with about 4 c c ether in separatory funnel. The extracted filtrate, still containing some ether, was injected in 0.5 c c dose intravenously in Rabbit 14, 700 gm. Dead in 30 seconds with marked convulsions and cries.

This extracted filtrate was kept at 5 C. over night and then placed in a vacuum to remove all the ether. When 0.5 c c was injected intravenously in Rabbit 15, 700 gm., it produced little effect. In 6 seconds the respirations went to 38 per 10 seconds but very shortly fell to normal.

The light amber colored jelly removed by the ether was dried, redissolved in 4 c c NaCl solution and centrifuged clear. Rabbit 16, 700 gm., received 0.5 c c intravenously; no symptoms.

The Lethal Agent Can Be Removed by Centrifugal Force.—Made up standard rabbit lung extract from perfectly fresh lung. This was centrifuged at 3,000 revolutions for 10 minutes = A.

The supernatant fluid A was recentrifuged in a special type of separator at 36,000 revolutions per minute for 15 minutes. This last supernatant fluid was perfectly clear but tinged with hemoglobin = B.

The fine sediment on the sides of the separator was washed off with 0.9% NaCl solution = C.

Rabbit 36, 1,190 gm., 0.3 c c of A intravenously; respirations accelerated; survived.

Rabbit 37, 1,080 gm., 0.5 c c of A; as 36.

Rabbit 38, 820 gm., 1 c c of A; dead in 2 minutes.

Rabbit 39, 800 gm., 2 c c of B; no symptoms.

Rabbit 40, 1,020 gm., 9 c c of B; dead in 1 minute.

Rabbit 36 was reinjected 15 minutes after the first inoculation with 0.3 c c of a mixture of equal parts of B and C; it scratched its nose and feet in one minute but showed no other symptom.

It is evident from these results that centrifuging at 3,000 revolutions per minute reduces the lethal action of the extract to about one-half that present when the centrifuging is performed at 1,200 or 1,500, and that 36,000 revolutions per minute brings about a still further reduction. The fine particles which are thrown out are either the lethal agents themselves or adsorb the lethal agent. Microscopic examination of the clear fluid, obtained at 36,000 revolutions, showed the presence of scattered isolated particles about 0.5 microns (estimated) in diameter and small aggregations of a dozen or so of these; the sediment was composed of isolated and massed bodies like those

described and of larger spherical refractile bodies 1.5-2 microns in diameter (estimated) and of aggregations of particles of both types. These granules did not stain well with Loeffler's methylene blue but stained with carbol gentian violet. Many of the large and some of the smaller particles gave a fat reaction with sudan III. None were acid-fast when stained by the tubercle method.

Morphin and Atropin Do Not Lessen the Lethal Action.—1. Rabbit 36, 1,190 gm., 0.08 gm. atropin sulphate slowly intravenously; then 0.5 c c lung extract; fell over in 15 seconds, dead in 30 seconds. Marked tremor of muscles.

Rabbit 37, 1,080 gm., 100 mg. atropin sulphate slowly intravenously and one minute later 0.5 c c lung extract. Dead in one minute. Symptoms typical.

2. Rabbit 41, 1,500 gm., ¼ grain (0.016 gm.) morphin sulphate intravenously and when lying passive it received 0.5 c c lung extract. Dead in one minute, convulsions after rolling on side.

SUMMARY

An extract of finely ground rabbit or guinea-pig lung in 0.9% NaCl solution is fatal for these animals when injected intravenously in a suitable dose. Rabbit lung is more fatal for the rabbit than for the guinea-pig and vice versa. Many of the symptoms of anaphylactic shock are produced—the acceleration of the respiration and peripheral irritation, both best shown by sublethal doses, the inspiratory dyspnea, the convulsions, the tolerance produced by graded repeated doses, the delay in the coagulation of the blood, all make one think of a possible relationship to anaphylaxis, but the lungs collapse on opening the thorax. Atropin does not protect. Morphin does not protect. Similar extracts of rabbit liver, kidney, ileum and spleen were harmless for rabbits.*

The lethal agent will not pass Berkefeld filter N at 600 mm. pressure. It is removed by animal charcoal. It is not extracted by ether. It can be removed by centrifugal force (36,000 revolutions per minute) and is apparently composed of, or adsorbed to, particles visible under the microscope.

By beginning with a sublethal dose, and then injecting at short intervals thereafter larger doses, a rabbit will survive, without symptoms, several lethal doses, just as in Besredka's and Friedberger's methods of producing "antianaphylaxis."

* Since we are unable to continue this work at present we wish to add some further observations to the proof of this article. Lung tissue may be autoclaved at 15-20 pounds pressure for 15 minutes and then ground up and extracted as usual without appreciably altering its lethal action. Boiling the extract itself renders it innocuous. Next to lung tissue we have found omentum most toxic. Kidney extract will kill in a dose of 2 c.c. Muscle and brain extracts produced no symptoms in doses up to 2 c c. An inoculation of muscle or brain extract given about an hour before a fatal dose of lung or omentum extract protected the rabbits. The toxicity of normal and pneumonic human lung extracts for some rodents and dogs has been reported upon by Rosenow; Solis-Cohen, Weiss and Kolmer (Jour. Inf. Dis., 1918, 22, p. 476), but from a somewhat different viewpoint.

It does not seem likely that the lethal agent comes from the interaction of lung tissue and homologous red blood cells, for incubating these together for a short time does not increase the poisonous action. Washed lung and thoroughly washed lung tissue fragments when ground up still yield particles which produce the symptoms and give protection. These particles can be removed by centrifugal force. A very similar toxic action was obtained by DeKruif[1] in repeating earlier work on the toxicity of homologous normal serum obtained by defibrination. The work of F. G. Novy and his pupils and the still more recent work of Kritchewsky[2] seems to throw the problem of anaphylactic shock into the field of colloid chemistry. While we are not entirely convinced that we are dealing with a particle phenomenon, we are inclined to favor the idea that colloidal particles are responsible for the lethal action. It still remains to be proven whether these particles have adsorbed to them something like an "internal secretion" capable of stimulating respiration.

[1] Jour. Infect. Dis., 1917, 20, p. 717.
[2] Ibid., 1918, 22, p. 101.

FOOD ACCESSORY FACTORS (VITAMINS) IN BACTERIAL CULTURE. II.

David John Davis

From the Department of Pathology and Bacteriology, University of Illinois, College of Medicine, Chicago.

In a previous paper[1] I called attention to the rôle that hemoglobin plays as a possible food accessory substance in the culture of hemophilic bacteria. It was shown that while these organisms in hemoglobin mediums attain a certain development, a much greater growth occurs when fresh animal or plant tissue is added to the medium or another bacterium is allowed to grow on the same plate. It would seem that there are at least these two food factors which tend to enhance the growth of the hemophil. These facts naturally suggest that the effect of the tissues on the plate cultures is dependent on substances comparable or analogous to the so-called vitamin substances that play an important rôle in animal metabolism.

The problem of finding substances which might enhance the growth of other bacteria has been undertaken. In the tests recorded in this article, the following bacteria were used: B. coli, B. typhosus, B. diphtheriae, Streptococcus hemolyticus, Staphylococcus aureus, Blastomycetes, Sporotrichum schenckii, Streptothrix, B. pyocyaneus, and B. prodigiosus.

In the first place, the effect of hemoglobin on these various organisms was tested. It is known that only a few organisms besides B. influenzae absolutely require hemoglobin. I had occasion some time ago[2] to review the facts. They are all small gram-negative bacilli. Other virulent bacteria like streptococci, pneumococci, meningococci, and gonococci grow well on rich blood medium to which other animal fluids like serum or ascites fluid is added. In my experience in growing the various bacteria of this type I have not noted that hemoglobin fluids are superior to other body fluids in this respect.

The ten organisms were inoculated on blood agar and serum agar 1 to 5 in each case and the series allowed to grow side by side, 8 succes-

Received for publication March 25, 1918.

[1] Davis: Jour. Infect. Dis., 1917, 21, p. 392.

[2] Davis: Jour. Am. Med. Assn., 1915, 64, p. 1814.

sive transfers being made at intervals of 2 to 3 days. They all grew well and the two series yielded growths that to the eye were quite alike in amount. Unlike the true hemophils then these organisms are not apparently specifically stimulated to grow through the addition of hemoglobin to ordinary mediums.

Pure hemoglobin 1% was next added to tubes of distilled water which were inoculated with the various organisms. In such tubes the organisms vary in the amount of growth that develops. B. diphtheriae and streptococci did not grow well. It was doubtful whether any growth occurred in the tubes and continuous transfers with these bacteria was not possible. The other bacteria all evidently showed some growth but it was not abundant and did not compare with growth on ordinary mediums containing peptone and albumin.

Since the presence of typical vitamin substances causing definite effects in man and animals exist in the bran of such grains as rice and wheat, tests were now made to determine whether or not the presence of these bodies in any way influenced bacterial growth. The medium consisted of 0.5% NaCl and 1% agar to which was added 5% by weight of each of the following substances: Polished rice flour, unpolished rice flour, pure white wheat flour, and whole wheat flour. The medium was heated at 100 C. for from 1-2 hours and then without filtration tubed and slanted. The 10 organisms were inoculated into each of the mediums, placed in the incubator, and each day the growth was carefully observed as to quantity. Transfers were made at intervals of 3 or 4 days through 10 generations for the purpose of testing the viability of the organisms when thus transferred; such observations actually extending over a period of 38 days.

All of the organisms grew on the mediums and none died out during the period of observation. They grew equally well on all 4 varieties, no appreciable differences being noted. The growth was not profuse, on the whole being less than on ordinary plain meat extract agar which was used as a control.

In the second series, polished rice, unpolished rice, and wheat bran were each added to ordinary plain meat extract agar and the organisms tested as before, transplants being made every few days. Careful observation from day to day on these cultures did not reveal any appreciable increase in the profuseness of the growth as compared with the growth in tubes not containing the grain products. In this respect the 2 sets of experiments were quite in accord.

B. diphtheriae and S. hemolyticus grew poorly on all the mediums containing grain products. The fungi — Sporothrichum schenckii, blastomycetes, and a pathogenic streptothrix, on the other hand, grew about equally well on all the varieties of medium. The growth was quite rapid, profuse and especially was pigment formation abundant and intense. In this respect these mediums are far superior to ordinary agar and even, especially in intensity of pigment production, to Sabouraud's 4% maltose agar. No differences were noted, however, between the growth on the medium containing the bran or hulls and that not containing it. Therefore, it would seem that the vitamin substances of grains, important in the prevention of beriberi and related diseases, are not of significance in promoting or influencing the growth of the organisms studied.

Another series of experiments were made using mediums containing the sprouted grain as a constituent. Rice and wheat were moistened and allowed to germinate until sprouts were about 1 cm. or more long. They were now dried and ground into a fine flour and then used in 5% solution exactly as was the flour in the previous experiments. This sprouted grain medium was decidedly superior to the unsprouted. All bacteria grew more rapidly and more profusely, including the fungi, which also yielded pigment in abundance on this medium. Several factors may be responsible for this. The sprouting process is associated with enzyme activity resulting in the solution of starch and in its change to sugars; also a change in the proteins with the formation of more soluble nitrogenous substances including amino-acids and related compounds. The presence of such substances no doubt furnishes a most excellent medium for bacteria. It has been observed by Holst and Froelich, Funk, and others that sprouted grain will prevent certain deficiency diseases simulating scurvy in animals and which results from feeding dry grains like oats, wheat, etc.; it is suggested that in the germination of the seed, substances are formed which act as antiscorbutics. The question therefore arises whether such factors may not play a rôle in the results I obtained and, at least in part, possibly explain the increased development of the organisms on this medium.

Hopkins[3] has observed that vitamins are substances readily adsorbed, and more recently Dorothy Lloyd[4] has emphasized this in connection with observations on the growth of meningococci; filtration

[3] Jour. Physiol., 1912, 44, p. 425.

[4] Jour. Path. and Bacteriol., 1916, 21, p. 113.

through filter paper or cloth will largely remove these bodies from solutions, but glass wool does not appear to adsorb them.

In the experiments in which sprouted wheat grain was used, portions of the medium were filtered several times through layers of filter paper and other portions were not. It was noted that on the whole the unfiltered part yielded decidedly the more abundant growth. It was especially noticeable in the cultures of the more profusely growing organisms like B. coli and staphylococci, less noticeable though evident in the case of cultures of B. diphtheriae and streptococci. The filtered part from the sprouted grain yielded growths of bacteria which though less profuse than on the unfiltered medium was without question superior to that obtained on mediums made with the unsprouted grain. This was evident in all the tubes without exception and is no doubt due to the presence of constituents rendered soluble by the sprouting process. These facts are therefore quite in accord with the observations on the adsorbability of certain of the growth factors in the medium and would point to vitamin substances in the sprouted grain medium as playing a rôle in growth stimulation.

SPOROTRICHOSIS FOLLOWING MOUSE BITE WITH CERTAIN IMMUNOLOGIC DATA

Plate 4

Josiah J. Moore and David John Davis

From the Department of Pathology and Bacteriology, University of Illinois, College of Medicine, Chicago

Sporotrichosis occurs spontaneously in the horse, mule, dog, rat, and man. The life history and the mode of transmission of the sporotricha are not well known. The disease practically always follows an injury to the skin, usually a puncture wound or an abrasion on an extremity. Lutz and Splendore[1] in Brazil observed the disease in rats and thought it was commonly transmitted from animal to animal through bites and scratches on the tail or extremities. A number of interesting cases have been reported following injuries received from the bites of lower animals. Recently the literature on the relation of animal and human sporotrichosis has been thoroughly reviewed by K. F. Meyer.[2]

Sutton[3] reported a case following a hen bite on the back of the hand. The wound later suppurated under home treatment. About 4 weeks later abscesses began to form on the forearm, no other part of the body being affected. Clinically this case was typical sporotrichosis but cultures evidently were not made. Microscopic examination of the contents of the abscesses revealed no organisms.

Olsen's[4] case was one in which the primary lesion appeared on the dorsum of the hand. No reference is made to previous injury but the statement appears that the patient had handled and killed many gophers, some of which were afflicted with sores.

Jeanselme and Chevallier[5] report an interesting case in a woman who developed nodular, gummatous sporotrichosis of the arm following the bite on the thumb by a white rat. This rat had been artificially inoculated with Sporotrichum jeanselme and was suffering from the experimental infection in various parts of the body. The nodules appeared on the arm nearly 3 months after the bite. The lesions were typical. The sporo-agglutination test was positive with Sporotrichum beurmanni at 1/300. A strong local intracutaneous test was obtained and a marked general reaction followed the subcutaneous injection of the killed organisms. The fixation test was negative.

Received for publication March 25, 1918.

[1] Centralbl. f. Bacteriol., I, O., 1917, 45, p. 631.

[2] Jour. Am. Med. Assn., 1915, 65, p. 576.

[3] Ibid., 1910, 55, p. 2213.

[4] Ibid., 1912, 59, p. 941.

[5] Bull. et mém. Soc. méd. d. hôp. de Paris, 1911, 31, p. 287.

Rispal and Dalous[6] report a case which is difficult to analyze. In 1877 the patient was operated on for phlegmon which followed the bite of a horse. It reappeared 10 years later and in 1909 there was still an old cicatrix. Here there developed multiple nodules which on examination proved to be an infection with Sporotrichum beurmanni.

de Beurmann and Gougerot[7] reported a patient seen by Rouslacroix who was bitten on the finger by a small dog. In 4 or 5 days the finger became painful, slightly inflamed and swollen. In about a month the skin ulcerated and discharged pus. Twenty-seven days after the bite, the first nodules developed on the hand and arm. The organism isolated from the lesions was Sporotrichum beurmanni. Another case of Rouslacroix reported in 1913 by de Beurmann and Gougerot, was attributed to the bite of a parrot.

In none of these cases can it be stated with certainty that the sporotricha were actually carried into the lesion by the teeth or that they were present in the mouth of the animals at the time. They may have been on the skin of the patient or may have later found their way into the wound, the bite simply serving as an atrium as any other puncture wound might do.

The patient whom we had the opportunity of observing, a boy, aged 13, was bitten on the index finger of his left hand by a field mouse about Sept. 10, 1916. This occurred while he was spending his vacation near Belfield, N. D., on the Little Missouri River. He had caught the mouse in a flax field and while playing with it received the bite. The wound bled at the time but was apparently healed in a few days. About 2 weeks later he noticed a swelling of the finger at approximately the point of the old bite. This increased until the entire terminal phalanx was swollen, reddened and hard but not very painful. By October 13 it had "come to a head" and was opened with a needle. There was considerable discharge of yellow pus which continued for several days, later becoming more watery in consistency.

October 19 a painless nodule was discovered on the arm some distance above the wrist. Two days later he visited a physician who enlarged the opening on the finger, cleaned it with carbolic acid and applied a wet dressing. No improvement followed, the nodules continuing to increase in size and number and November 5 the patient consulted Dr. Yeakel of Chicago, who thinking the disease resembled blastomycosis, referred the patient to us for bacteriologic examination.

Cultures were made from the lesion on the finger November 6 and 8. The material was obtained from the lesion by first removing the slough and scab and then passing a sterile platinum loop deep down to the base and under the margins of the ulcer. The granulations here were abundant and bled readily. Many leukocytes were present. Tubes of various mediums were inoculated and placed at both incubator and room temperature. After a few days all of the tubes yielded growths, several a pure culture of an organism suggestive of Sporotrichum schenckii. Transfers were made to various other mediums and several pure cultures of the organism were thus obtained.

November 11 a large nodule above the wrist was aspirated and cultures and smears were likewise made of the thick mucoid, honeylike pus obtained.

6 Arch. de dermat. et de syph., 1909, 12, p. 689.

7 Les Sporotrichoses, 1912, p. 278.

No organisms could be found in the smears by direct examination but colonies exactly like those from the finger appeared on the cultures after 5 days.

The growths after several days were white and after about 1 week the growths especially on sugar mediums began to turn brown and soon became quite black. The surface became corrugated with rather deep irregular grooves and there was some growth into the depth of the medium. After numerous transfers on artificial mediums after a period of several months no important change has been noted. The strains still continue to produce a deep black pigment.

Microscopically the growth consists of a dense feltwork of septate branching mycelial filaments with hyphae having many terminal and lateral spores more or less oval and pear-shaped. They are gram-positive and in every way correspond to the morphology of Sporotrichum schenckii. Chlamydospores in old cultures are found in moderate numbers.

Fig. 1.—Initial lesion following a mouse bite on the finger.

White rats were inoculated with suspensions of the culture, some intraperitoneally, some subcutaneously. The former developed typical pearly gray nodules on the omentum and about the stomach, spleen and testicles. Two, after several weeks, showed nodular swelling on their hind legs and died. On necropsy the typical lesions of sporotrichosis of the long bones of the leg were found and cultures made from these bone lesions yielded pure growth of the sporotrichum. All the animals not treated with potassium iodid died after from 2-3 months.

The rats inoculated subcutaneously developed local lesions which later broke down forming ulcers from which the sporotrichum was repeatedly grown. Smears of the exudate from these lesions and also from the peritoneal nodules showed in abundance the characteristic elongated tissue forms. No branching or mycelial growths were found in the tissues. To several of the rats with lesions following the inoculations, potassium iodid was given subcutaneously in doses of 0.05 gm. The rats with cutaneous lesions responded promptly, the ulcers in the course of a few days showing marked improvement. A lesion,

the size of a dime, on 1 animal, just above the tail was entirely healed in about 3 weeks. Before this treatment was begun the lesions following subcutaneous inoculation of sporothrix had been steadily increasing in size.

When we first saw the patient the terminal phalanx of the left index finger was swollen, very firm and of a dusky red color. Two irregular shaped ulcers about 0.5 cm. in length were present on the ventral surface from which a few small drops of thick yellowish pus could be expressed. These ulcers are shown in the accompanying photograph (Fig. 1). The first subcutaneous nodule about 0.5 cm. in diameter was situated at the base of the index finger on its inner aspect. There were 15 others varying in size from 0.5-2 cm. scattered along the course of the lymphatics on the back of the hand, the radial side of the forearm and the arm to about 5 cm. below the axilla. Most of them were firm, painless, and with little or no discoloration over the surface. The largest nodule, located about 5 cm. above the wrist was softer than the others and its surface was dark blue or violet in color. Softening was undoubtedly beginning in this lesion. From this nodule was aspirated the thick yellowish mucoid pus from which the pure cultures described were grown. The regional lymph glands were not enlarged. No other lesions were found at this time; a general examination showed the patient to be in all other respects a normal, well developed boy. His past and family histories, having no direct connection with the disease, are omitted.

Generally the patient feels quite well; his temperature for over 2 weeks has been normal; he has never had chills. The urine was normal. Leukocyte counts varied from 9,000-10,500; hemoglobin 90%; erythrocytes 5,000,000; neutrophils, 65%, small lymphocytes, 27%, large lymphocytes, and transitionals 6.5%, eosinophils 1%, and basophils 0.5%.

November 11, 15 grains of potassium iodid daily were administered and gradually increased to 30 grains a day. Within 10 days the ulcers were crusted over, no more pus was formed but the nodules appeared slightly larger and were all more or less discolored. November 23, a bluish nodule 1 cm. in diameter was found about 5 cm. above the elbow on the inner surface of the right arm. This had probably been present for some time but had been overlooked. At this time the finger had almost regained its normal size and was healing rapidly. The nodules were decreasing in size. The treatment (30 grains of potassium iodid daily) was continued for 2 months at which time the finger was apparently healed; the nodules on the left arm had disappeared with the exception of a small one on the back of the hand, but the nodule on the right arm could still be palpated.

We did not see him again until February 24, when he presented the following condition. The end of the finger is swollen, the scar tissue is reddened with four small crusted areas scattered over it. A nodule about 1 cm. in size has appeared on the calf of the left leg. The patient states that he discontinued the potassium iodid for 1 week after we last saw him (January 16) and that when he recommenced taking it he had such gastric distress that he stopped the medicine permanently. About a week ago the scar of the old ulcer broke open and some thick yellowish pus exuded. Since that time pus in small amounts has discharged from the finger each day. During this time he noticed new nodules appearing and the one on the hand growing larger.

He was immediately given 30 grains of potassium iodid daily. After 10 days the condition was again much improved. The nodule on the right arm became soft and on incising about 4 drops of a yellowish mucoid pus, similar to that obtained from the finger and the nodule on the other arm were expressed. Cultures and smears were made at once. No organism could be

found in the latter but the former yielded pure growths of sporotricha after 5 days, demonstrating beyond question that this nodule was sporotrichotic. This lesion entirely healed in 2 weeks but remained somewhat discolored. With the continuance of the specific treatment all the lesions practically disappeared within 1 month.

We did not see the patient again until Aug. 23, 1917, when he presented himself with an open ulcer at the base of the index finger. He had discontinued potassium iodid some months previously. He stated that this "sore" had been discharging for about a month. A watery, yellow pus could be forced out but cultures made from this contained only staphylococci. No subcutaneous nodules were present and the patient felt in the best of health. October 10, the condition was the same but the cultures this time yielded not only staphylococci, but also a few colonies of sporotricha. Cultures October 23, also yielded Sporotrichum schenckii.

He now commenced with potassium iodid again and continued for 3 weeks. During the early part of this period the sinus was washed twice a week with tincture of iodin and with Lugol's solution. This caused profuse discharge which continued for a few days. The abscess closed but reopened the latter part of December when he recommenced taking potassium iodid. Cultures were made Jan. 15, 1918, but contained only staphylococci. There was no discharge from this date until February 23, when a few drops of pus were obtained, potassium iodid having been taken during the entire period. The abscess, which measured about 1.5 cm. and was dusky red when first seen, measured about 1.8 mm., was not discolored and had apparently a dense connective tissue wall. Roentgen-ray pictures in October demonstrated that the lesion was not associated with bone or joint involvement. At the present time there is a discharge of a drop or two of pus every 2nd day and the lesion is apparently slowly healing, due probably to the larger intake of potassium iodid which has been increased to 60 minims daily.

The symptoms presented here are typical of the disseminated subcutaneous gummatous type of sporotrichosis. They include the characteristics emphasized by Gougerot; numerous lesions beginning in the form of painless nodules with partial softening and without marked impairment of the general condition; mucoid or citron yellow pus; cold indolent swellings; no enlargement of the regional lymph glands. A point which he considers most characteristic, the formation of abscesses from the softened nodules with violet colored margins, did not appear. This may have been on account of the early diagnosis and recourse to iodid therapy.

An important point is the dissemination of the infection to various portions of the body as shown by the occurrence of nodules on the opposite arm and on the left leg. The probable routes of infection were the lymph or the blood stream. Blood cultures were sterile in this case, but it is known that the organisms appear in the blood stream at times in human sporotrichosis. In experimental sporotrichosis in rats positive cultures from the heart blood are readily obtained in advanced

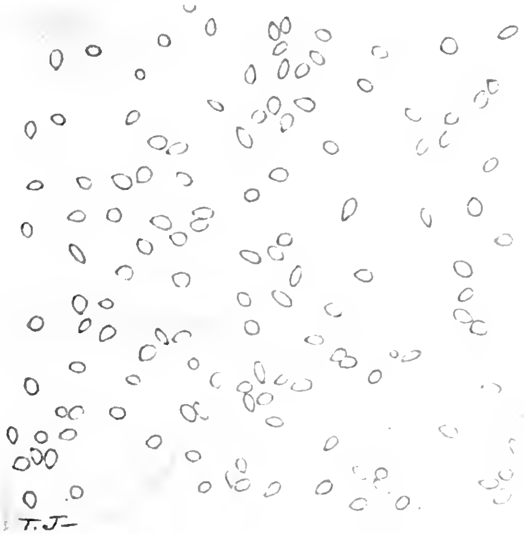


Fig. 2.—Sporo-agglutination. Control serum 1-40.

Fig. 3.—Sporo-agglutination. Patient's serum 1-40.

stages of the infection. There were no demonstrable lesions of the internal organs at any time in this patient.

Potassium iodid in this case was quite specific for the disease and confirms previous observations that it is one of the best examples of a specific therapeutic agent known. However, as illustrated by the repeated recurrences in this case, the drug must be given in moderate or large doses consistently and for some time after all lesions and symptoms have disappeared.

From time to time the serum of the patient was tested for the presence of agglutinins. The homologous as well as French and American strains were used; also the original Schenck-Hektoen strain of Sporothrix schenckii. The technic was as follows:

The suspension was made by macerating in a mortar with salt solution some of the tough growth several weeks old from an agar slant and then straining it through either a coarse filter paper or a plug of cotton. The mycelial filaments are held back on the filter and a pure suspension of spores is obtained which may then be diluted to the proper degree. A suspension containing about 200-400 spores in a high power field is a convenient one to use. A small amount of formalin added to the suspension will kill the organisms but does not interfere with the test.

The macroscopic test we have not found suitable since the large heavy spores soon settle out in both specific and normal serum suspensions. A convenient way to obtain a definite clumping is to make the various dilutions in small tubes and allow to stand for several hours or over night. The tubes are then shaken and hanging drop preparations made at once. The spores under the influence of the serum soon begin to clump and the reaction is usually a very definite one. The final reading is taken after 24 hours. Figure 3 shows this phenomenon of spore agglutination microscopically. The results are tabulated in Table 1.

It will be noted that in the various tests agglutination appeared at fairly uniform dilutions, these being 1/160 or occasionally 1/320. Furthermore, the agglutination titer was practically uniform for the various strains, no appreciable differences being noted between the homologous strain, a strain obtained from France (Sporotrichum beurmanni) and the original Schenck-Hektoen strain. This is in accord with the results obtained by one of us,[8] using immune rabbit serum. They also confirm the results of Wilder and McCullough[9] who tested various strains of sporotricha with serum from a case of sporotrichosis of the eye acquired accidentally in the laboratory. Their highest titer was 1/64.

Controls of blood from various diseases including syphilis and blastomycosis gave no clumping in 1/20 dilutions.

[8] Davis, D. J.: Jour. Infect. Dis., 1913, 12, p. 140.

[9] Jour. Am. Med. Assn., 1914, 62, p. 1156.

The agglutination test was first made on November 15 approximately 2 months after the bite and about 1 month after the appearance of nodules. At this time it was distinctly positive. November 23 it was again distinctly positive and indeed at slightly higher dilutions than in the previous test. December 15 and also December 30, tests were made again with similar results. Following the recurrence in January and February an agglutination test was again made February 26 which gave a distinct reaction at 1/320. No further tests were made until August 10 at which time the test was nearly negative there being only a slight clumping at 1/20. In the meantime the nodules had disappeared under proper therapy though there was still a small open ulcer at the base of the finger which, as stated, at this time yielded only staphylococci.

Later, however, when the iodid therapy was discontinued by the patient and the discharge from the finger increased, cultures made from the pus again yielded sporotricha. In all probability a few organisms had been present continuously but were not detected by the culture in August. The discharge continued from the finger and at the present time (March, 1918) still persists

TABLE 1

SPORO-AGGLUTINATION OF VARIOUS STRAINS WITH PATIENT'S SERUM ON NOVEMBER 23

Strains	Serum	1-20	1-40	1-80	1-160	1-320	1-640
Homologous	Patient	++	+−	−−	+	0	0
	Normal	0	0	0	0	0	0
Equine	Patient	++	+−	+	+	0	0
	Normal	0	0	0	0	0	0
Human (American)	Patient	+−	−−	+	+	+	0
	Normal	0	0	0	0	0	0
Human (France, de Beurmann)	Patient	+−	−−	+	−	0	0
	Normal	0	0	0	0	0	0
Human (Schenck-Hektoen)	Patient	++	++	+	+	0	0
	Normal	0	0	0	0	0	0

and cultures made from time to time have yielded sporotricha. Agglutination tests made Jan. 14, 1918, and Feb. 26, 1918, were again definitely positive at dilutions of 1/80.

Complement fixation tests were made simultaneously with the agglutination tests. This infection has been noted by others to produce a high content of specific complement fixing substances.

The antigens for fixation were prepared in the same manner as for the agglutinins except that after filtering 0.5% phenol was added as a preservative. Four sporothrix strains were used in the tests, one from de Beurmann, the original Schenck-Hektoen strain, one isolated from a horse by Meyer, and the strain isolated from the patient. For control antigens we used one prepared in a similar manner from a strain of blastomycosis, three syphilitic antigens, and a gonococcus antigen. After suitable tests for anticomplementary action on the sporothrix and blastomyces antigens, one-third to one-thirtieth of the anticomplementary dose was used in the tests. Three-tenths c c of the equine,

de Beurmann, and the patient's antigens, and 0.2 c c of the Schenck-Hektoen antigen gave complete hemolysis. As small amounts as 0.01 c c of the various antigens caused complete fixation when added to 0.1 c c of the patient's serum. The chicken hemolytic system employing the technic previously described by one of us[10] was followed in all the tests. As control serum we used in every test a positive Wassermann serum, serum from several cases of blastomycosis and from various other diseases, and normal serum.

The patient's serum reacted specifically to the sporothrix antigens and was negative in all the control antigens during the first few months of the infection. Likewise the control serums gave complete hemolysis with the sporothrix antigens. Although it is a well known fact that a positive syphilitic serum will often give a nonspecific positive reaction

TABLE 2

COMPLEMENT FIXATION IN SPOROTRICHOSIS WITH VARIOUS ANTIGENS

Dec. 12, 1916

Antigen	C C	Serum	Result
Original Schenck-Hektoen........	0.01	Patient 0.1 c c	No hemolysis
Original Schenck-Hektoen.......... ..	0.02	Patient 0.1 c c	No hemolysis
Original Schenck-Hektoen	0.1	Normal 0.1 c c	Complete hemolysis
Original Schenck-Hektoen............	0.2	0	Complete hemolysis
French (de Beurmann)................	0.01	Patient 0.1 c c	No hemolysis
French (de Beurmann)..	0.02	Patient 0.1 c c	No hemolysis
French (de Beurmann)................	0.1	Normal 0.1 c c	Complete hemolysis
French (de Beurmann)................	0.3	0	Complete hemolysis
American (Davis).....................	0.01	Patient 0.1 c c	No hemolysis
American (Davis).....................	0.02	Patient 0.1 c c	No hemolysis
American (Davis).....................	0.1	Normal 0.1 c c	Complete hemolysis
American (Davis).....................	0.3	0	Complete hemolysis
Autogenous.....................	0 01	Patient 0.1 c c	No hemolysis
Autogenous...........................	0.02	Patient 0.1 c c	No hemolysis
Autogenous..............	0.1	Normal 0.1 c c	Complete hemolysis
Autogenous............	0.3	0	Complete hemolysis
Blastomyces..........................	0.2	Patient 0.1 c c	Complete hemolysis
Blastomyces..........................	0.5	0	Complete hemolysis
Syphilitic................	0.1	Patient 0.1 c c	Complete hemolysis
....	....	Patient 0.2 c c	Complete hemolysis

with bacterial antigens all the syphilitic serums tested were negative. The blastomycotic serums always resulted in complete hemolysis with all antigens including the blastomyces. This concurs with the work of B. Davis[11] and others who were unable to demonstrate any specific fixation substances for blastomycosis. The antigens and serums used, together with the results of a typical test, are presented in Table 2. Here approximately 200,000 spores sufficed to fix the complement with the antibodies in 0.1 c c. of serum.

It was noted that the Schenck-Hektoen antigen possessed more anticomplementary substances than the other strains which naturally suggested a larger content of spores. Counts of the approximate number of spores in each

[10] Moore, J. J.: Jour. Infect. Dis., 1916, 18, p. 569.

[11] Jour. Infect. Dis., 1911, 8, p. 190.

antigen shortly after their preparation was as follows: de Beurmann 18 million, patient 21 million, Schenck-Hektoen 37 million per cm., thus confirming our supposition. It is an easy procedure to count the spores using the red field of a hemocytometer chamber and allowing 10-15 minutes for the spores to settle. This led us to believe that we might standardize a sporothrix antigen by estimating the number of spores in the solution. We therefore prepared an antigen by carefully touching a platinum loop to the surface of an old culture so as to get spores and but few mycelia and then transferring these spores to a small amount of normal salt solution containing 0.5% phenol. Such an antigen, containing 46 million spores per c c was tested with the Schenck-Hektoen antigen, then about 6 weeks old, and containing at that time approximately 500,000 spores per c c. The latter antigen, however, tested much stronger. Since the Schenck-Hektoen preparation undoubtedly contained some of the material from the macerated hyphae, mycelia and spores, and when first prepared contained less spores than the so-called spore antigen but reacted in smaller amounts when first prepared, it would appear that a crushed mixture would give the best antigen. The autolysis of the spores may increase the strength of the antigen as the antigenic property may be due to this factor. Further tests, however, did not confirm this.

A so-called spore antigen was carefully prepared as described and the same culture from the patient was ground up as a "triturated" antigen. They were diluted until each contained approximately the same number (30 million) of spores per c c. They were tested at once so as to give no opportunity for autolysis. Serums drawn at different times were used. The results indicate that there is little difference between the "spire" and "triturated" antigens; that approximately 2,000,000 spores will fix the antibodies; that the antibodies will persist in the serum when sealed and on ice for a period of 6 months. We have lately tested serum 13 months old and find little apparent decrease in complement fixing substances. In addition we tested antigens of the various strains which had been kept for 9 months and although the spores had decreased from 19 to 25 million to 1,500,000 and less per c c they still retained their antigenic value, indicating that the antigenic properties lie in substances which may act before or after the autolysis of the spore.

As the infection subsided under proper therapy there appeared to be a gradual decrease in the antibody content of the serum of the patient. Tests made with serum taken Dec. 30, 1916, did not show fixation of complement as strongly as with serum taken at earlier periods although all were tested at the same time with equal amounts of the antigens. After the recurrence in February, 1917, the reaction was as strong as before. In the following August, with none of the antigens could we demonstrate complement fixing bodies (Table 2), though the different sporothrix antigens were of nearly equal strengths, that is, contained approximately a like number of spores. From October, 1917, to March, 1918, the antibodies were found in as large amounts as in February, 1917.

When prepared by the same technic there was no apparent difference in the fixing ability of the various strains of sporotricha tested.

Intradermal injections of 0.1 c c of sporotrichin preparations of the de Beurmann and Schenck-Hektoen strains with control of a like amount of normal salt were made November 27 in the right arm. The sporotrichin was prepared like the agglutination suspensions, then sterilized in the autoclave for 1 hour and kept in the icebox. A normal individual was treated in the same manner as the patient.

After 48 hours the arm of the patient presented the following appearance: At the point of injection of the de Beurmann antigen was a small raised pustule about 5 mm. in diameter, surrounded by an elevated, angry, indurated, hyperemic area about 1.4 cm. in diameter which in turn was encompassed by an area of secondary hyperemia of lighter hue with an indistinct margin and which was about 5.5 cm. in diameter. The appearance of the Schenck-Hektoen injection site was the same, the measurements being 1.4 cm. for the primary hyperemia and 5 cm. for the entire area. Surrounding the normal salt control was a reddened area 1.3 cm. in diameter. The measurements of the areas on the arm of the normal individual were: de Beurmann 5 mm., Schenck-Hektoen 5 mm., normal salt 3 mm.

During the first 24 hours after the tests, the patient complained of axillary tenderness but no enlarged glands could be palpated. After about 36 hours he was nauseated and had a headache. His temperature after 48 hours was 99.2 F. The axillary glands were tender and slightly swollen. This was when the intracutaneous reactions were at their height. The erythema gradually disappeared, the skin became brownish in color and desquamated after several days. The arm of the patient regained its normal appearance after 7 weeks, the arm of the control in 3 weeks. March 7, 3 weeks after the recurrence a second test, using the de Beurmann sporotrichin, a blastomycin and normal salt in 0.1 c c amounts, was made. Within 24 hours the patient complained of headache, malaise and nausea, and had axillary tenderness. Within 48 hours the sporotrichin test measured 7 cm. x 7.5 cm., with a central pustule 4 mm. in size. The blastomycin test was 6 mm. in diameter at its maximum and brownish red in color; the salt injection was 2 mm. in diameter. The axillary lymph glands were palpable. A comparison of the reactions at their maximum is shown in Table 3.

Since it is now recognized that potassium iodid administered by mouth may increase the severity of certain intracutaneous tests,[12] and as the control was not receiving potassium iodid, we decided to test several normal persons while they were taking this drug. The drug was administered by mouth in doses of 10 grains 3 times a day for several days. On the 7th day the skin tests were made with equal amounts (0.1 c c) of the same material and measurements made at the end of 48 hours. One control, a patient with blastomycosis, received intracutaneous injections of the de Beurmann and patient's sporotrichin when getting nothing. Later while taking potassium iodid he

[12] Kolmer, Immermann, Matsunami and Montgomery: Jour. Lab. and Clin. Med., 1917, 21, p. 401.

was again tested with the de Beurmann strain and with a blastomycin prepared from his own organism. In one normal control the injections were made before and also while the person was receiving potassium iodid. In the others the first tests were made while receiving potassium iodid and repeated again 6 weeks after the drug had been discontinued. All these control intradermal tests yielded negative results (Table 3).

TABLE 3

THE INFLUENCE OF POTASSIUM IODID ON THE SPOROTRICHIN INTRACUTANEOUS REACTION

Subject	Antigens 0.1 c c	Diameter in M M of Zone of Reaction	
		Without Potassium Iodid	Receiving Potassium Iodid
Sporotrichotic patient	de Beurmann	55 × 45	55 × 53
Sporotrichotic patient	de Beurmann	....	75 × 70
Sporotrichotic patient	Schenck-Hektoen	50 × 43	52 × 50
Sporotrichotic patient	Schenck-Hektoen	....	30 × 25
Sporotrichotic patient	Patient	55 × 45	33 × 24
Sporotrichotic patient	Blastomycin	....	8 × 6
Sporotrichotic patient	Salt solution	....	12 × 10
Sporotrichotic patient	Salt solution	....	4 × 2
Blastomycotic patient	de Beurmann	5 × 4	9 × 8
Blastomycotic patient	Patient	7 × 6	....
Blastomycotic patient	Blastomycin	....	9 × 8
Blastomycotic patient	Salt solution	3 × 2	1 × 1
Normal I	de Beurmann	5 × 5	9 × 7
Normal I	Patient	5 × 4	9 × 8
Normal I	Salt solution	2 × 2	3 × 2
Normal II	de Beurmann	....	6 × 6
Normal II	Patient	9 × 8	12 × 10
Normal II	Salt solution	....	2 × 1
Normal III	de Beurmann	....	6 × 5
Normal III	Patient	11 × 7	6 × 6
Normal III	Salt solution	....	2 × 2
Normal IV	de Beurmann	....	5 × 4
Normal IV	Patient	10 × 8	6 × 5
Normal IV	Salt solution	....	1 × 1

As shown by this table the sporotrichin intradermal reaction is specific. It is not only greater in degree and severity, but it is accompanied by general symptoms of malaise and rise in temperature. There is apparently no difference in reaction between the de Beaurmann, the Schenck-Hektoen strains and the patient's strains. Blastomycin has no diagnostic value in blastomycosis and produces only a normal reaction in sporotrichosis. Potassium iodid does not interfere with the diagnostic value of sporotrichin.

In August, 1917, we had an opportunity of testing the patient at a time when he was not taking potassium iodid and had taken none for 4 months. It was at this time that he had a discharge from the base of the index finger from which only staphylococci were isolated. Of each of 3 sporotrichins, the de Beurmann strain, the Schenck-Hektoen strain, and the patient's strain, 0.1 c c were injected. The measurements at the end of 48 hours were de

Beurmann 5.5 x 4.5 cm., Schenck-Hektoen 5 x 4.3 cm., and patient 5.5 x 4.5 cm. His temperature at the end of 24 hours was 99.4 F. during which time he complained of headache and could not work. At this time complement fixation tests as stated above were negative. Agglutination tests made with different strains were likewise practically negative.

That the sporotrichum was causing the ulcer was confirmed by positive cultures obtained at later dates. These tests would suggest that the skin reaction is more delicate than the other tests. However, tests made in January, 1918, using as antigens the patient's and the Schenck-Hektoen strains, measured 2.5 cm. x 3 cm. and 2.4 x 3.3 cm. The patient stated that with the exception of a headache he had none of the general symptoms produced by his former intracutaneous reactions. It is difficult to determine whether the antigenic property had been reduced in this time, as the antigens were the same as those used in all the previous allergic tests, or whether the reaction was decreasing. Supposing that this was not changed and finding as we did complement fixation and agglutinin bodies were high at this time it would appear that the immunity tests are not always parallel in a quantitative way. The findings of August, 1917, which were almost directly the contrary, would also suggest this.

SUMMARY

An instance of an infection with Sporotrichum schenckii following the bite of a field mouse in North Dakota is described. It is not known whether the organisms came from the mouse or from the soil or the skin.

The infection has now persisted for about 18 months, improving with administration of iodid and relapsing when the iodid is discontinued.

Repeated sporo-agglutination tests gave positive results at serum dilutions varying from 1-320 to 1-80. At one time, when the patient had almost recovered, the test was slight at 1-20, but later became stronger. In general this test parallels the clinical course.

Complement fixation tests made at various times generally yielded definitely positive results, corresponding thus to the agglutination tests. In the icebox the antibodies in the serum retain their activity for over one year.

The antigens in these tests consisted of several strains of sporotricha including the autogenous organism, the original Schenck-Hektoen strain, a French strain (Sp. beurmanni) obtained from Gougerot, an equine strain from Meyer in the United States, and a

strain previously isolated from a typical case in this country. All behaved approximately alike toward the patient's serum, pointing to the identity of the different strains.

Intracutaneous tests made with French and American strains on this patient yielded strongly positive reactions.

A blastomyces antigen did not cause a reaction in this patient nor did a blastomycotic patient react to the sporotrichum antigen.

The antigens are stable substances resisting heat and retaining their activity for over a year.

The administration of potassium iodid to sporotrichotic or normal persons does not appreciably alter the sporotrichin reaction.

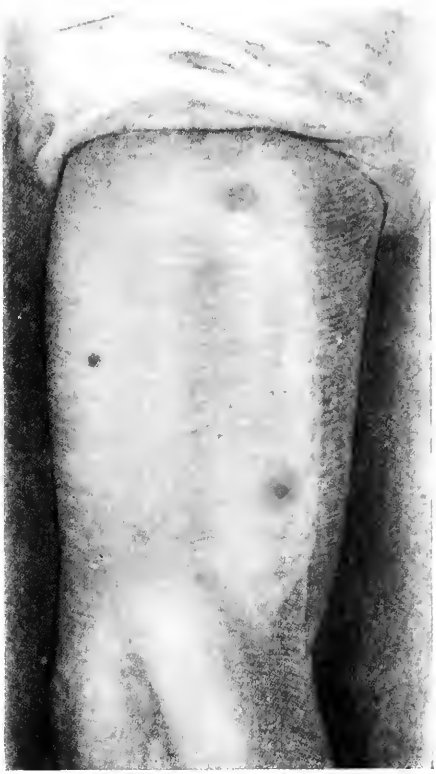
In sporotrichum infections antibodies of various kinds are readily generated. This disease, therefore, is in marked contrast to blastomycosis, a closely related disease, in which antibodies are apparently generated with great difficulty or not at all.

EXPLANATION OF PLATE 4

Fig. 1.—Cutaneous reactions on normal person. Upper, sporotrichin; lower, blastomycin; left, salt solution.

Fig. 2.—Cutaneous reactions on sporotrichosis patient. Upper, sporotrichin; lower, blastomycin; left, salt solution.

PLATE 4



1

2

THE ROUTINE CULTIVATION OF TUBERCLE BACILLI FROM THE SPUTUM BY PETROFF'S METHOD

H. J. Corper, L. Fiala, and Lincoln Kallen

From the Laboratories of the Municipal Tuberculosis Sanitarium, Chicago

A method for cultivating the tubercle bacillus from discharges, excreta, and tissues which would give a large percentage of positive results in positive cases and would not consume too much time would be of inestimable value. It would open up new avenues for study and classification of the tubercle bacillus, for finding its virulence (as derived from various sources) and probably also for study of the immunologic phases of tuberculosis. A method recently described by Petroff[1] offered many advantages over previous methods for the isolation of the tubercle bacillus, and seemed worthy of an exhaustive trial especially as a routine procedure. The method consists briefly of two steps: The destruction of many of the rapidly growing micro-organisms by means of sodium hydroxid, and the inoculation of the resultant material on an egg medium similar to the one introduced by Dorset, but containing gentian violet in concentration of 1 : 10,000 to inhibit the growth of the contaminating organisms which were not destroyed by the sodium hydroxid. The preparation of the medium consisting of whole egg, beef or veal juice and gentian violet was described by Petroff as follows:

Meat Juice: 500 gm. beef or veal are infused in 500 c c of a 15% solution of glycerol in water. Twenty-four hours later the meat is squeezed in a sterile meat press and collected in a sterile beaker.

Eggs: Sterilize the shells of the eggs by immersion for 10 minutes in 70% alcohol or by pouring hot water on them. Break the eggs into a sterile beaker and after mixing the eggs well, filter through sterile gauze. Add 1 part by volume meat juice.

Gentian Violet: Add sufficient 1% alcoholic gentian violet to make a dilution of 1 to 10,000. Tube about 3 c c in each sterile test tube and inspissate for 3 successive days; on the 1st day at 85 C., until all the medium is solidified, changing the places of the tubes if necessary; on the 2nd and 3rd days for more than 1 hour at 75 C.

Received for publication, April 6, 1918.

[1] Jour. Exper. Med., 1915, 21, p. 38.

[2] Am. Med., 1902, 3, p. 555.

Fresh sputum is advisable. Equal parts of sputum (about 5 c c) and 3% sodium hydrate are well shaken and left in the incubator for 20-30 minutes until the sputum is fairly well digested. The sputum is then neutralized to sterile litmus paper with normal hydrochloric acid, centrifugalized and the sediment inoculated into several test tubes containing the mediums described in the foregoing. Neutralization is not necessary but is advisable.

Petroff obtained 69 positive cultures from 69 specimens of sputum, 6 of those being negative by direct microscopic examination, but having been positive for tubercle bacilli at some time. In a final report on the use of this method he[3] was able to isolate and cultivate the tubercle bacillus from 129 sputums of 135 specimens. In a brief article on the use of the Petroff method Mitchell and Simmons[4] report that good growth was obtained from 28 of 35 specimens of sputum, all positive by smear, while 6 were contaminated and 1 revealed no growth.

In a preliminary report on the use of Petroff's method of isolating the tubercle bacillus, Keilty[5] calls attention to the importance of neutralization after the use of the sodium hydroxid, avoiding drying of the culture and keeping the incubator temperature between 37 and 38 C. and finally reports[6] having examined 25 sputums of which 18 were positive by smear, 12 of which gave growth; of the 7 negative by smear 1 showed growth. Of the 12 positive cases, 4 were obtained in pure culture and 8 showed microscopic evidence of growth (being overgrown by contamination later). Of the 4 pure cultures, 2 averaged 10-14 organisms to a field on the original sputum examination and 2 showed 1 to several fields. Of the 18 positive sputums, only 4 had many organisms to a field and 2 of these gave pure cultures while 1 had microscopic evidence in culture. Of the 4 pure cultures, 1 case showed 2 contaminations of 4 tubes inoculated. The other 3 grew without contamination. The first appearance of gross growth averaged 14-21 days. Soparkar[7] was able to isolate tubercle bacilli from sputum that was 20 days old by the Petroff method and found that even 2½% sodium hydroxid at 37 C. up to 3 hours did not kill tubercle bacilli. He gave no figures on his number of positive findings, but reports he was able to grow the bacilli by this method even when animal inoculation and the microscope failed to find them. Stewart[8] used the original Petroff method on 37 specimens of sputum positive by microscopic examination and obtained growth from 24, or 64.8%. The shortest time required for growth was 2 weeks (in comparison to 7-14 days observed by Petroff) while the average period was 3 to 4 weeks and in some instances colonies could not be detected in less than 6 weeks after inoculation. The organism was recovered in 69.2% of samples of milk (13 samples were infected, 4 with human [all gave growth] and 9 with bovine [5 gave growth]) that were artificially infected.

[3] Petroff, S. A.: Bull. Johns Hopkins Hosp., 1915, 26, p. 276.
[4] Jour. Am. Med. Assn., 1915, 65, p. 245.
[5] Jour. Exper. Med., 1915, 22, p. 612.
[6] Ibid., 1916, p. 41.
[7] Indian Jour. Med. Research, 1916, 4, p. 28.
[8] Jour. Exper. Med., 1917, 26, p. 755.

The investigations in this paper were made with a twofold purpose, to obtain cultures of tubercle bacilli for the study of their virulence and to gain information on the various steps involved in the technic of cultivating tubercle bacilli according to the method devised by Petroff. In order to determine whether the acidity of the medium is of any practical significance in determining the growth of tubercle bacilli, as Petroff and Dorset[9] both paid no particular attention to the reaction of the egg medium, the bacilli were grown on 5% glycerol agar of varying acidity with the results indicated in Table 1.

TABLE 1

GROWTH OF TUBERCLE BACILLI ON GLYCEROL AGAR OF DIFFERENT REACTIONS

Reaction*	Alkaline			Neutral	Acid			
	2.0	1.0	0.5		0.5	1.0	2.0	4.0
No. 1305—Recently isolated virulent human bacillus	—	+++	+++	+++	+++	+++	—	—
Bovine—Old laboratory strain	—	++	+++	+++	+++	+++	—	—

The results given are the readings made after 1 month incubation at 37 C. averaging 4 tubes in each case.

* The reaction given is in the terms of normal alkali or acid using phenolphthalein as indicator.

It is to be noted that on 5% glycerol agar a good growth of tubercle bacilli is obtainable within wide range (from 1.0+ to 1.0—) of reaction of the medium. Szaboky[10] found the range of reaction on egg mediums also to have wide limits, the best growth occurring on strongly acid mediums, but strongly alkaline reaction or neutrality not preventing fairly good growth.

Likewise the range of use of glycerol was wide as was indicated by the original investigations of Nocard and Roux[11] and also by the following experiment: A series of tubes of Dorset egg medium were prepared containing none and varying amounts of glycerol and these were then inoculated with recently isolated virulent human tubercle bacilli and an old laboratory bovine strain (Table 2).

[9] Am. Med., 1902, 3, p. 555.

[10] Centralbl. f. Bakteriol., 1907, I, O., 43, p. 651.

[11] Ann. de l'Inst. Pasteur, 1887, I, p. 19, and Centralbl. f. Bakteriol., 1887, 2, p. 136.

From these preliminary experiments it is to be noted that the glycerol content or the acidity of the mediums within wide ranges (1.0+ to 1.0—) play no important part in the cultivation of tubercle bacilli.

Petroff in his first contribution lays great stress on the use of meat juice—beef or veal—and on inspissation in the preparation of the egg medium. In order to determine whether ordinary bouillon containing beef extract and peptone could not be substituted for the meat juice and whether higher temperatures, as for instance autoclaving instead of inspissating the medium, would not answer the purpose the following experiment was made.

TABLE 2

Effect of Different Glycerol Content on the Growth of Tubercle Bacilli on Dorset's Egg Medium

	Glycerol per cent				
	0	1	2	4	6
1769*	—	++	++	++	++
1907	—	++	++	++	+
461	—	++	++	++	+
1584	—	++	++	++	++
1885	—	++	++	++	++
924	—	++	++	++	+
1769	—	++	++	++	++
Bovine†	—	++	++	++	++

* The numbers indicate recently isolated human tubercle bacilli.
† An old laboratory bovine strain obtained from Dr. Paul A. Lewis and accustomed to growing on glycerol agar.
The results are the readings of 2 tubes inoculated, observations made 1 month after cultivation at 37 C.

Four culture tubes of gentian violet egg medium were inoculated with sputum (previously treated with 3% sodium hydroxid as directed by Petroff) one of these prepared exactly according to the directions of Petroff and inspissated, the second a duplicate but autoclaved instead of inspissated, a third prepared by mixing one-fourth volume of neutral (to litmus) 20% glycerol broth of 4 times ordinary broth strength (prepared with sodium chlorid, Liebig's beef extract and Witte or Difco peptone) and 3 volumes of mixed white and yolk of egg to which finally is added 1 : 10,000 gentian violet (Grübler) and inspissated, and a fourth duplicate of the third in ingredients but autoclaved.

The autoclaving is carried out by slanting the tubes in a horizontal self sealing autoclave (Duparquet, Huot and Moneuse Co.), rapidly expelling the air and replacing it by steam before the culture tubes have had time to warm up, shutting all valves and outlets quickly and maintaining a steam pressure of 10-15 lbs. for 30 minutes to 1 hour.

The growth of the tubercle bacilli obtained from 63 microscopically positive sputums by seeding a single tube of the 4 differently prepared mediums resulted as shown in Table 3.

A glance at Table 3 reveals that in cultivating tubercle bacilli on Petroff's medium practically the same results can be obtained regardless of whether the medium is prepared from fresh beef juice as recommended by Petroff or from Liebig's beef extract and peptone as carried out in the ordinary laboratory preparation of mediums. Autoclaving, which is a more rapid procedure, also does not possess any disadvantages as compared with inspissating of the egg medium. It may also be noted that no macroscopic cultures were obtained within 2 weeks' time.

TABLE 3

COMPARISON OF GROWTH OF TUBERCLE BACILLI FROM POSITIVE SPUTUMS ON MEDIUMS PREPARED ACCORDING TO PETROFF'S DIRECTIONS, INSPISSATED AND AUTOCLAVED, AND ON THE SAME MEDIUMS SUBSTITUTING BROTH PREPARED FROM LIEBIG'S BEEF EXTRACT AND PEPTONE FOR MEAT JUICE

Amount of Growth		Readings	Slight Macroscopic Growth†	Good Moderate Macroscopic Growth	Excellent Heavy Macroscopic Growth
Petroff's Egg Medium	Inspissated 43 Negative	4*	1 1* 1**	4**	1
		6	1 1**	1-3**	1
		8	4	1	
	Autoclaved 46 Negative	4	1 1* 3**	13**	1
		6	2*	2	1
		8	1	1	
Egg Medium Prepared with NaCl, Liebig's Beef Extract, and Peptone	Inspissated 46 Negative	4	0	1**	
		6	3* 2**	1-3**	1
		8	3	2	1
	Autoclaved 43 Negative	4	1-2* 1**	3**	1
		6	1-1* 3**	1 1**	1
		8	1	2	

Observations of the cultures were made at 2, 4, 6, and 8 weeks after inoculation. Since in no case was there a visible macroscopic growth after the 2 weeks, this interval is not included in the tabulation.

† A figure without an exponent indicates that the growth occurred as indicated on the chart but remained stationary throughout the 8 weeks observation period. A figure with an * as exponent indicates that the growth was noted as slight and became a moderately good growth. A figure with a ** as exponent indicates that the growth was noted as tabulated either as a slight or moderately good growth and became an excellent heavy growth.

These results were practically corroborated in an experiment in which 20 sets of tubes were seeded directly from 20 freshly isolated cultures of human tubercle bacilli.

It is also noteworthy that the scrupulous care recommended in preparing the eggs and meat juice is redundant, since not a single case of inherent contamination was obtained during the course of these investigations. Thorough hot air sterilization of all culture tubes, however, was a precaution observed. Before inoculation of the completed medium it was incubated (precautions being taken against drying) for at least 4 days to rule out contamination from this source.

In order to gain information on the efficiency of the routine cultivation of tubercle bacilli from the sputum by Petroff's method and also to obtain cultures of bacilli for other studies, 526 fresh sputums were cultivated using 2 tubes of Petroff's gentian violet medium for seeding each sputum. These sputums were all microscopically positive for tubercle bacilli after being stained by the ordinary carbolfuchsin method. From these 526 sputums there were obtained 144 positive macroscopic (27.3%) cultures of varying grades of intensity. The results are recorded in Table 4 from the standpoint of the individual sputum, in Table 5 from the standpoint of the individual culture tube, and in Table 6 from the standpoint of the number of bacilli found in the original stained sputum.

TABLE 4

Results of the Cultivation of Tubercle Bacilli from the Sputum Considered from the Standpoint of the Individual Sputum

Positive Cultures			Negative Cultures	Contaminated Cultures
+	++	+++		
22	47	75	100	282
Totals	144 (27.3%)		382	

If a sputum gave no growth in 1 tube and contamination in 1 tube, it was recorded under negative cultures.
If a sputum gave a growth in 1 tube and no growth or contamination in the second, it was recorded under the proper division of growth.
When growth occurred in 2 tubes from 1 sputum the best growth was recorded.

The conclusions to be drawn from Tables 4, 5 and 6 are briefly:

In cultivating tubercle bacilli from the sputum by Petroff's method but using only 2 culture tubes for seeding and macroscopic growth as criterion of positive findings there were obtained, from 526 sputums, 144 growths (27.3%). These results in no way vitiate the higher percentage obtained by other observers, notably Petroff, in smaller series in which more culture tubes were seeded, recultivation of contaminated cultures carried out, and microscopic growths considered. There was noted no relationship between the number of bacilli found in the original sputum examined microscopically after staining and the percentage positive growths obtained or the intensity of their growth.

In the continuation of cultures which have been isolated Petroff calls attention to the fact that the egg gentian violet medium is not always suitable for this purpose since perpetuation of the culture on

this results in loss of power to grow with a simultaneous staining of the seeded material by means of the gentian violet. In order to note the effect of successive seeding of freshly isolated cultures of virulent human tubercle bacilli a series of 25 well grown cultures obtained from human sputum were transferred in each case to 2 fresh tubes of Petroff's gentian violet egg medium 2 successive times. The following results were obtained in these experiments.

TABLE 5

RESULTS OF THE CULTIVATION OF TUBERCLE BACILLI FROM THE SPUTUM CONSIDERED FROM THE STANDPOINT OF THE INDIVIDUAL CULTURE TUBE

	Positive Cultures			Negative Cultures	Contaminated Cultures
	+	++	+++		
	36	66	89	212	649
Totals	191 (18.1%)			861	

Of the 25 well grown cultures 9 gave good growths on both tubes seeded, 7 gave good growth on 1 tube and the other revealed a slight growth with 3 of these, 2 were contaminated (it is noteworthy that Petroff cultures which have been allowed to remain in the incubator, frequently develop contaminating growths even after good growths of tubercle bacilli have occurred and contaminations also occur on transplants which can only be attributed to the development of organisms from the original sputum which were merely held in abeyance by the sodium hydroxid treatment and 1 : 10,000 gentian violet; early transfers to fresh mediums reduce the possibility of development of these contaminators), and 2 revealed no growth; 2 gave slight growth in two tubes, 1 slight in one tube and negative in the other, and 6 were negative in both

TABLE 6

CLASSIFICATION OF CULTURES OBTAINED FROM POSITIVE SPUTUMS DEPENDENT ON THE NUMBER OF BACILLI FOUND IN THE ORIGINAL STAINED SPUTUM

	Growth							No Growth							Contaminations						
	Slide			Field				Slide			Field				Slide			Field			
	1-5	6-25	Over 25	1-5	6-25	26-100	Over 100	1-5	6-25	Over 25	1-5	6-25	26-100	Over 100	1-5	6-25	Over 25	1-5	6-25	26-100	Over 100
	4	4	5	52	53	6	20	5	1	0	44	46	10	4	20	3	2	129	82	26	20
Totals	144							100							282						

If a sputum gave no growth in 1 tube and contamination in 1 tube, it was recorded under negative cultures.

If a sputum gave a growth in 1 tube and no growth or contamination in the second, it was recorded under growth.

tubes. Of 14 good growths which were seeded a second time, 5 gave good growths in two tubes, 3 in one tube and the other was contaminated in two instances and dried out in the third; 6 were negative, 2 of these contaminated in 2 tubes and 4 in one tube.

In the majority of the tubes in which growth failed to occur and no contaminations were found the original masses of tubercle bacilli seeded were stained with gentian violet, in this corroborating Petroff's findings.

SUMMARY

The routine cultivation of tubercle bacilli from microscopic positive sputum by Petroff's method, seeding 2 gentian violet egg medium tubes from each sputum, resulted in obtaining positive macroscopic cultures of varying grades on primary culture from 144 (27.3%) of 526 sputums. There was noted no relationship between the number of bacilli found in the original sputum and the percentage of positive growths obtained or their intensity.

The glycerol content or acidity (as pointed out by Szaboky, in experiments with egg medium) of the medium within wide ranges are of little importance in the determination of growth.

Egg medium prepared as directed by Petroff with fresh beef juice revealed no particular advantages over mediums in which was substituted sodium chlorid, beef extract, and peptone as used in ordinary broth. The inspissation of the Petroff gentian violet egg medium on 3 successive days also proved to have no advantages over a single autoclaving. Petroff's findings that the gentian violet egg medium is not suitable for the continued cultivation of tubercle bacilli was corroborated (24%, 6 of 25, failing to grow on 2 tubes in the first seeding and 43%, 6 of 14, failing to grow on 2 tubes in the second seeding).

FURTHER EXPERIENCES WITH BRILLIANT GREEN AGAR AND OTHER PLATING MEDIUMS FOR THE ISOLATION OF TYPHOID AND PARATYPHOID BACILLI FROM FECES

Charles Krumwiede, Jr., Lawrence A. Kohn, Anne G. Kuttner, and Elsie L. Schumm

From the Bureau of Laboratories, Department of Health, New York City

Since describing a method of preparing a brilliant green agar plating medium for the isolation of typhoid and paratyphoid bacilli from feces,[1] the number of specimens examined has reached a total of over 7,000. It seems advisable therefore to present the results obtained as well as slight modifications of the methods employed as dictated by the experience gained in handling this large material. Since our report other observers have suggested various plating mediums for fecal examinations, and these have been tried sufficiently to determine their practical value. Our opinion as to their merits, as based on our experience, is also given.

As we have modified the preparation and standardization of our medium somewhat and to avoid the inconvenience of reference to our former report, it seems advisable to describe the medium and the methods as now employed.

Preparation of Medium.—The composition of the agar base is as follows:

Liebig's Extract of Beef	3 gm.
Peptone	10 gm.
NaCl	5 gm.
Agar	15 gm.
Water	1 liter

The agar is dissolved in half the water by autoclave, the other ingredients in the remainder of the water by heating on a gas stove. After complete solution the two halves are mixed and the reaction adjusted by the addition of alkali. The final reaction desired is the neutral point of the Andrade indicator. This indicator is prepared by adding 16 c c of N/1 NaOH solution to 100 c c of a 0.5% aqueous solution of acid fuchsin. The alkali changes the red of the fuchsin to orange or yellow. Mediums adjusted to the neutral

Received for publication April 8, 1918.

[1] Krumwiede, C., Jr., Pratt, J. S., and McWilliams, H. I.: Jour. Infect. Dis., 1916, 18, p. 1.

point of this indicator are pink when hot and colorless when cold, which corresponds to between 0.6% and 0.7% acid to phenolphthalein (hot titration). The reaction of the agar may be adjusted to the phenolphthalein reaction given or directly adjusted to the Andrade indicator by adding 1% of the indicator to a small measured volume and determining how much alkali is needed to bring it to the point desired, namely, pink when hot and colorless when cold. After adding the appropriate amount of alkali, thus determined, to the bulk of the medium, the latter should be retested and readjusted if necessary. The Andrade indicator adjusts itself somewhat slowly to changes in reaction; a little time therefore should be allowed to elapse between each addition of soda. We have found that with Fairchild's peptone and Liebig's extract that the addition of 8 c c of normal soda per liter will give a satisfactory end reaction. This will vary depending with the brand of peptone and extract employed, but once the necessary amount of soda is determined, the reaction can be closely approximated by employing this amount in succeeding batches of medium, avoiding in this way the necessity of titration. After adjusting the reaction, the agar medium is cooled, beaten egg white added and then boiled and filtered. After filtration the entire preparation is mixed thoroughly to insure uniformity of the whole product, bottled 100 c c to a bottle and autoclaved for 30 minutes at 15 lbs. pressure.

At the time of use the needed number of bottles are melted and to each 100 c c of agar is added 1 c c of the Andrade indicator, 1% of lactose, 0.1% of glucose and finally the appropriate amount of brilliant green. For the addition of the carbohydrates it is most convenient to prepare a stock solution in distilled water, containing 20% of the lactose and 2% of glucose, sterilizing the solution by steaming in the Arnold sterilizer. Five c c of this solution added to 100 c c of agar will give approximately the percentages required. The brilliant green is added from a stock 0.1% solution in distilled water. Two concentrations of brilliant green are employed, the appropriate amounts being determined by preliminary standardization (see below under standardization). The medium is mixed, poured into plates so as to give a rather thick layer allowing 6 plates for every 100 c c of medium. The finished plates must be dry and this is best assured by using porous earthenware covers. With these, the medium can be covered as quickly as poured.

Standardization.—This is the difficult step and if not successfully carried out, much or all of the value of the medium is lost. In our previous report we advised the use of the Rawlings strain of B. typhosus for standardization. This strain, however, has fluctuated so much in its susceptibility to brilliant green that its use has been discontinued. Freshly isolated cultures have given somewhat better results but positive stools, if obtainable, are by far the more reliable material for this purpose.

The stock brilliant green solution will keep for about a month. A fresh solution therefore should be employed in standardizing a new batch of agar and the batch of medium should be as large as possible so that the number of standardization tests will be reduced to a minimum.

As each new batch of medium is received plates are poured using 3 dilutions of the dye, namely, 1 : 200,000; 1 : 330,000, and 1 : 500,000. For convenience these are expressed in terms of the fractions of a c c of a 0.1% solution of brilliant green added to a 100 c c of agar; the amounts are, respectively, 0.5, 0.3, and 0.2 c c. The plates are then inoculated with equal amounts of material, spreading it by means of a spatulum. A nonrestraining medium such as Endo is used as a control. The best material for standardization are positive stools from typhoid carriers or cases. If obtainable, several fecal specimens should be inoculated to compensate for the variability of the fecal flora, slight variations in resistance of different strains of B. typhosus, and to average the variables due to technic. If only one positive stool is available this should be plated in duplicate or used to inoculate suspensions of 2 or 3 normal stools which are then used for inoculation. The suspensions should be comparatively light so that the resulting plates will have discrete colonies, otherwise comparison by count or estimate will be difficult or impossible.

The stools should be freshly collected as the persistent types in older stools tend to be the dye-resistant types and are therefore not satisfactory as a measure of the restraining action of the dye on the average fecal flora. If positive stools are not available, suspensions of fresh normal stools may be inoculated with broth cultures of recently isolated typhoid strains and the plates inoculated. The addition of a loop to 0.1 c c of an 18-hour broth culture to 10 c c of stool suspension of moderate density will give a well balanced mixture of B. typhosus and fecal bacteria. Pure cultures of B. coli should not be used to determine the restraining action of the dye. The degree of restraint of the average fecal flora is the fact wanted.

After incubation the plates are inspected and based on the results, selections made of the dilutions to be used. Two dilutions are chosen: first, the greatest amount of dye which has little effect on the number and size of the typhoid colonies, but which shows a marked restraint of the fecal flora; second, a greater amount which shows a moderate reduction in the number and size of the typhoid colonies and usually a still greater reduction of the fecal flora. Slightly greater concentrations of dye are used in practice than would seem to be indicated by the standardization results as the inoculation will be heavier, thus reducing the activity of the dye to some extent. With most batches of mediums and with the sample of dye we employ the 2 optimum dilutions average 0.2-0.3 c c, respectively, of a 0.1% solu-

tion of the dye to 100 c c of agar. (See brands of dye.) The reasons and advantages of two concentrations of the dye are given later.

Interpretation of the results of standardization tests and the selection of the optimum dilutions are most easily and accurately determined by a graphic representation of the results. Averages are taken of the figures recorded for the different specimens on the Endo medium and on the dye agar plates according to concentrations taken and the following data charted: the average percentage reduction on the dye plates of (a) colonies of fecal bacteria other than typhoid, (b) typhoid colonies, and (c) reduction in size of typhoid colonies using 1 mm. as the standard. In the accompanying representative charts (Charts 1, 2, 3, and 4) the results with fecal typhoid and culture typhoid strains are given separately for comparison. The selections that should be made for routine use are indicated by a heavy vertical arrow. The curves show clearly the basis of selection. Charts 1 and 2 are representative of the general limits of fluctuation with different batches of agar with the dye we have used in our routine work.

The two dilutions selected according to this method serve well in routine work for the isolation of both B. typhosus and B. paratyphosus. When the latter is the only type to be sought, one strong concentration may be employed alone, as this type is much more resistant to the dye than B. typhosus. An advantage of using stronger concentrations in this case would be the greater degree of restraint of the accompanying fecal flora. With a standardization result indicating that 0.2 c c and 0.3 c c are satisfactory for the isolation of B. typhosus from 0.35-0.5 c c can be used for B. paratyphosus. During an outbreak of paratyphoid fever, where one strain is presumably common to all the cases and carriers, the medium can be directly standardized, using a positive stool or a freshly isolated strain as soon as one is obtained. Because of the greater resistance of B. paratyphosus, wider limits of error are allowable than in standardizing for B. typhosus, and freshly isolated cultures give very satisfactory results. We have recently employed this method with marked success in the case of a regiment infected with B. paratyphosus A.[2]

Adaptability of Various Brands of Dyes.—Various samples of dyes have been tried. Of these the Bayer, Grübler and Hoechst brands have given successful results and the dilution range of differential action was closely alike. Of the other samples, bearing no name but probably of German manufacture, some have been fairly good. Examples are given in Charts 3 and 4. Although

[2] Krumwiede, C., Jr.: Jour. Infect. Dis., 1917, 21, p. 141.

good differential action was obtained with both samples a considerable concentration was needed, indicating that there were inactive impurities present Although we have not personally encountered dye samples which, even when very dilute, inhibited B. typhosus as well as the fecal flora, we have had a report of such an experience. The results would seem to indicate the presence of highly toxic impurity.

Colony Characteristics.—On the medium, after 18 hours' incubation, the typhoid colonies will be found to be very characteristic. They

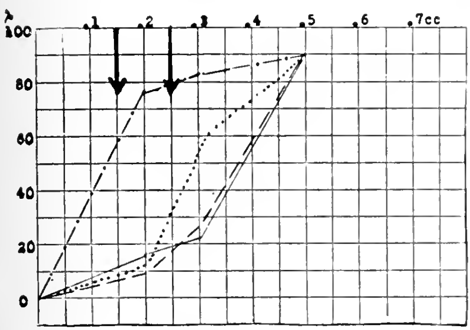


Chart 1.

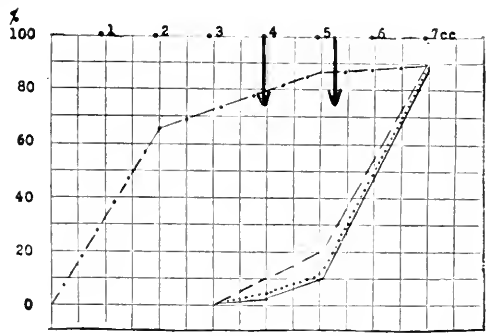


Chart 3.

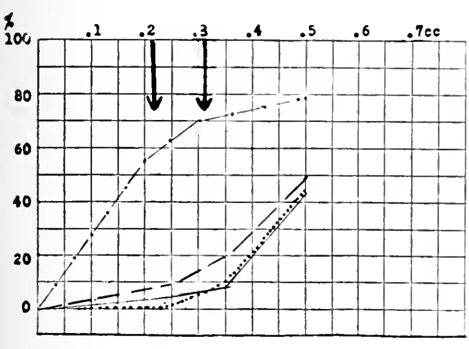


Chart 2.

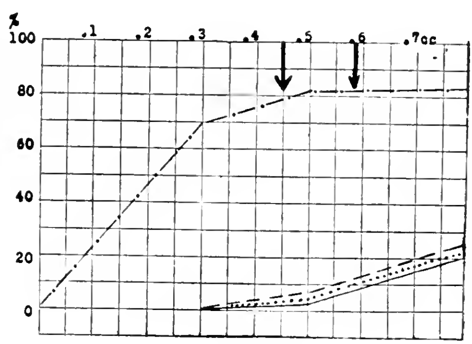


Chart 4.

Explanation of charts: —.—.—.— = Fecal types; ———————— = Fecal typhoid; .... = Culture typhoid; — — — — — — Size of typhoid colonies.

Charts 1 and 2.—Standardization test. Bayer dye.

Charts 3 and 4.—Standardization test. Dye? See under Brands of Dye.

are of good size (1-1.5 mm.); the glucose not only enhances their growth but is also the main cause of their characteristic appearance. Viewed through the plate against a dark background, the light passing obliquely through the agar, they have a peculiar striated, flaky appearance. With artificial light and a hand lens, under the same conditions

they have the appearance of a coarse wool fabric. They may take on a faint mauve tinge from fermentation of the trace of glucose, which accentuates somewhat their characteristic appearance. The larger colonies may resemble the paratyphoid B type. Colonies of this organism tend to be larger, heavier and more opaque. They are often tinted a delicate green, and the markings are less distinct, though still evident at the periphery. The paratyphoid A colonies resemble the typhoid colony more nearly than that of the paratyphoid B, but we have frequently observed an extremely flattened, slightly tinted colony whose edges melt into the agar. The markings of the colonies of all three organisms are usually more prominent on the stronger dye plates. The only organisms which simulate the typhoid colony are certain partially restrained colon types which may develop on weak dye plates. They present exaggerated markings, with distinct cross-bar striations, and they are usually completely excluded on the stronger dye plates. These organisms frequently agglutinate spontaneously in the saline drop (see below). Some of the dye-resistant "intermediates" develop paratyphoid-like colonies.

With no known change in the mode of preparation an occasional batch of agar will develop very large typhoid colonies. These luxuriant colonies tend to be much less characteristic in their markings, and the tendency to the development of this type should be noted when standardization tests are made, so that such colonies will not be overlooked in using the medium subsequently in routine work. With an agar of this kind slight inhibition by the dye will result in more typical colonies apparently due to a lessening of the density of the colony, thus making the markings more visible. It is possible even with the average batch of agar that the dye, exerting a very slight influence, contributes somewhat to the development of the typical colony.

The colonies of the fecal types which develop take on a moderate pink color if they ferment lactose, but the color change is not marked. Even without a sharp color change, they are so different from the typhoid or paratyphoid colony that the practiced eye passes over them without hesitation. This lack of sharp color change may seem a decided disadvantage to bacteriologists accustomed to the sharp color differences of Endo and similar mediums, on which the typhoid-paratyphoid colonies are presumably those showing no change of indicator. We have approached the problem, however, from the standpoint of giving the colonies of these types positive characteristics, rather than relying

on a negative characteristic, inability to ferment lactose. In this regard, the medium is unique, and, to our mind, the more logical way of attacking the problem of evolving a still more satisfactory medium.

Method of Inoculation.—To obtain discrete colonies various methods of inoculation are advised, as for instance the use of successive dilutions of an initial fecal suspension. Such a method takes time and glassware and is not very expedient in handling large numbers of specimens. With some practice one can obtain very satisfactory results by diluting the suspension to a density which experience has shown will yield sufficiently abundant but discrete colonies. This density corresponds roughly to a suspension of 1 part of solid feces to 15 parts of fluid. We dilute fluid feces or emulsify solid feces to this density using peptone water and allow the suspensions to stand 15-30 minutes to allow sedimentation of the particles and then inoculate the plates from the surface of the suspension. To save time inoculation is done with a double loop so that one or both may be emptied on the plate. For each specimen 2 series of plates are employed consisting of a weak and a strong dye agar and Endo plate. The fecal suspension is inoculated only on the dye plates, 1 loop being placed on each of the first series and 2 loops on each of the other series. The material is then spread with a bent nichrome wire, rubbing out the material on the weak plate, then on the strong plate and then passing to the Endo plate, the material adhering to the wire sufficing to inoculate this plate. The second series is then streaked in the same fashion. The stronger dye-plate in each series receives in this way the heaviest inoculation. The spreader is only sterilized between specimens.

Nichrome wire is a cheap and convenient substitute for platinum and a very satisfactory spreader is made by bending the wire to an angle of 120° and bending up the tip to avoid tearing the plates. It does not fuse well in glass and the shank is best bent in a zigzag fashion and pushed into narrow bore tubing with thick wall. Owing to its greater rigidity a finer gage of nichrome wire can be used than in the case of platinum, thus compensating for the slower cooling of the former. If 2 spreaders are used, 1 cools while the other is in use. We have found the wire spreader more convenient than glass rods which cool very slowly if sterilized by heat while inoculating, and are inconvenient to wash and sterilize in bundles especially when the number needed is large.

Identification.—Although the preceding gives the details of the medium it seems well to describe again the following methods because of their expediency and reliability in mass work. Preliminary identification of the colonies is done by means of the slide agglutination method using highly potent serum. A drop of highly immune serum in low dilution is placed on a slide, and beside it one of salt solution. The suspicious colony is picked off with a small loop of fine wire, and a sufficient amount rubbed off in the salt solution to give a slight clouding. Some of the growth remaining on the loop is then rubbed off similarly in the serum drop. An immediate clumping, visible to the naked eye is a positive result; spontaneous agglutination will be noted in the control. When the colony is suspicious of paratyphoid, 3 drops,

salt solution, A and B serum, may be used, a portion of the colony being rubbed in the salt-solution and one serum, and another portion in the second serum, with intermediate sterilization of the loop. It is essential, for this purpose, that the serum be of high titer, as a slow reaction is time-consuming and permits drying of the drop. In general, we prefer an older serum of original high potency, which has lost not only in total titer for its homologous type, but also in content of group and normal agglutinins. Under these conditions, the dilutions used may be as low as 1 : 50 to 1 : 100. With a known and tested serum, the results are very reliable; with an unknown serum, serious error may result because of group reaction. Occasionally a slight delay in clumping, due to the prezone phenomenon, will be noticed, but there is usually some indication of a reaction in the serum, and dilution with an additional loop or two of salt solution induces prompt and marked clumping. We have noted increased agglutinability in colonies on dye plates as compared with those on Endo. This is apparently due not to the dye alone but to the glucose as well; in any case, it is no disadvantage. Inagglutinable strains, showing no reaction in the concentrations of serum employed, are rarely isolated from feces, but a characteristic colony, nonagglutinating, should not be overlooked. In emergencies, with known serums and trained workers, the macroscopic slide agglutination could be considered final for all promptly positive cases with little likelihood of error. The doubtful slower reactions, usually few in number, would have to be verified by isolation. A negative slide agglutination is usually accepted as a final negative result, but we fish some suspicious colonies occasionally as a control on the serum. Positive results are checked by fishing to the Russell medium or better a modified Russell containing 1% of saccharose as well as 1% of lactose and 0.1% of glucose.[3] Paratyphoid-like types which may give some reaction with the paratyphoid serums are more surely excluded in this way, as many of these types ferment saccharose but attack lactose only slightly or not at all. If the reaction on the Russell medium is typical the results of the slide agglutination are verified by using the growth for macroscopic agglutination. If the serums used are of sufficient titer and the range of group and normal agglutinins is known, this suffices for all practical purposes. If the serums are not altogether satisfactory false positive results are likely to be encountered in examining for B. paratyphosus and the results are then best verified by determination of the cultural and fermentative characteristics.

[3] Krumwiede, C., Jr., and Kohn, L. A.: Jour. Med. Research, 1917, 37, p. 225.

If the search is to include the paratyphoid types causing food poisoning (B. cholerae suis and B. enteritidis) fishing of the suspicious colonies becomes necessary as well as the full determination of the cultural and fermentative characteristics and final identification by agglutination with the appropriate serums and possibly agglutinin absorption.

There is one source of error in the use of brilliant green agar which is common to all selective mediums. In fishing a suspected colony, impure cultures may be obtained, as fecal bacteria which have been inhibited are capable of multiplication when transferred to a favorable medium. This danger should be kept in mind, and suitable precautions taken. In fishing, it is well to avoid touching anything but the apex of the colony. It is well to restreak on an Endo plate if there are very few positive colonies in a crowded area and it is questionable whether a pure culture can be obtained.

Results Obtained with the Brilliant Green Medium.—Table 1 gives the numerical results in the isolation of B. typhosus from feces.

TABLE 1

FECAL EXAMINATION FOR B. TYPHOSUS

Source	Totals	Endo Positive	Dye-Agar Positive	Percentage of Endo Positives Compared with Total Positives
All sources	7,111	920	1,235	74.5
From carriers	594	502	564	89
All sources exclusive of carriers	6,517	418	671	62

As one would expect, the chronic carriers give the least increase in positive results as their feces usually contain a relative abundance of typhoid bacilli allowing of easy isolation with any nonrestraining medium. The fewer the bacilli in proportion to other fecal types the greater should be the expected increase of positive results with the dye-medium. This expectation is realized and shown in the results in Table 2.

TABLE 2

SPECIMENS FOR DIAGNOSIS FROM SUSPECTED CASES OF TYPHOID FEVER

Number	Endo Positive	Dye-Agar Positive	Percentage of Endo Positives Compared with Total Positives
115	18	45	40

Table 3 is of interest in this connection. The examinations were carried out in making a sanitary survey of an insane asylum because of the development of cases of typhoid fever. Nearly all of the positive results were obtained from patients found to be suffering from the disease. Because of their mental state the existence of the disease was unsuspected in some of the patients till positive fecal reports led to routine temperature taking. A rise of temperature was frequently the only indication of the presence of the disease.

TABLE 3
SPECIMENS, STATE INSANE ASYLUM

Number	Endo Positive	Dye-Agar Positive	Percentage of Endo Positives Compared with Total Positives
847	30	46	65

Although the figures indicate the numerical increase of positive results on the dye-agar they do not indicate the greater ease of isolation on this medium in cases in which the Endo medium is positive as well. With a dye-sensitive fecal flora as encountered in the average feces, the suppression of these types on the more heavily inoculated dye-plates results in the development of many more typhoid colonies as compared with the Endo plates or may result in the development of typhoid colonies alone. The contrast between the Endo and dye-plates in the actual number of typhoid colonies and the comparative number in relation to the total number of colonies is usually very striking.

We have continued the use of the Endo medium mainly to obtain comparative data and to some extent as a check on the use of the brilliant green medium because of a frequent change of personnel among those carrying out the actual examinations. With sufficient experience it could be omitted as we have encountered positive results on Endo with negative results on the brilliant green agar in only two instances. In one this seemed to be purely accidental; in the other the dye-plates were overgrown by B. aerogenes types, the feces being unusually rich in this dye resistant variety.

Two concentrations of dye are used for the following reasons: Brilliant green is an exceedingly sensitive colloidal restraining agent and its differential action is quantitative in nature, being elicited only at certain dilutions. The activity of the dye is not necessarily the same

in different batches of medium apparently prepared in an identical manner. The restraining action is elicited in higher dilutions in a clear than in a cloudy agar, and any foreign matter, a trace of cotton in the agar, for example, will reduce the activity of the dye. For this reason the restraining action will vary with the amount of fecal protein inoculated. Furthermore, the fecal flora of different specimens varies somewhat in dye sensitiveness. Strains of B. typhosus likewise vary slightly in their resistance. Two dilutions compensate for these variables and for any slight miscalculation in the selection of the dilutions as based on the standardization test. The stronger dye-plate has one advantage that, although there be a reduction in the number of typhoid colonies developing, the greater restraint of the fecal flora may bring them into greater relief. Table 4 gives the comparative results with the 2 strengths of dye; it does not show, however, the greater preponderance of B. typhosus on one or the other concentration which is frequently encountered in examining the plates from an individual specimen. This must be considered in interpreting the results given, especially the greater number of positive results with the stronger concentration of dye.

TABLE 4

FECAL EXAMINATION FOR B. TYPHOSUS: COMPARATIVE RESULTS ACCORDING TO STRENGTH OF DYE

Positive on weak dye-agar only	26
Positive on strong dye-agar only	81
Positive on both weak and strong dye-agar	1,128
Total positive on dye-agar	1,235
Total positive on Endo	920

The results in the isolation of B. paratyphosus (Tables 5 and 6) have been somewhat better than in the isolation of B. typhosus.

On the whole the results obtained have equaled those originally reported as based on only a relatively small series of examinations.

In our original communication,[1] we recommended Kendall's modification of Endo's medium because of the convenience of using the same agar stock for both this and the dye-agar. We have found, however, that although Kendall's modification gives excellent differentiation for the more vigorous lactose fermenters, a great many colonies which were absolutely colorless on this medium, ferment lactose rapidly when transferred to Russell's double sugar medium. We have tried numerous other modifications of Endo and have found the one recently recommended by Robinson and Rettger[4] to be the most

[4] Jour. Med. Research, 1916, 34, p. 363.

satisfactory, giving as it does the sharpest color to the lactose fermenting colonies with the least diffusion. We have changed their method slightly to avoid adding the indicator till the medium is to be used, as there is always the danger of the reduced color returning, especially if large batches of mediums are prepared at one time.

The medium is prepared as follows: Twenty-five gm. of agar, 10 gm. of peptone, and 5 gm. of meat extract to a liter of water, are dissolved by heating on a gas stove. The ingredients are made neutral to litmus by the addition of 10% solution of sodium carbonate. A further 10 c c of sodium carbonate is added per liter. The medium is cooled to 45 C. and one egg per liter added. Autoclave for 30 minutes at 15 lbs. pressure. Filter and add 1% lactose and bring to a boil to dissolve the sugar. Finally add another 10 c c of 10% solution of sodium carbonate per liter, the reaction should now be 0.3 — to phenolphthalein (hot titration). The medium is bottled in 100 c c amounts and autoclaved for 10 minutes at 10 lbs. pressure. To the melted agar just before use, add 0.5 c c of a saturated alcoholic solution of fuchsin and 1 c c of a 10% solution of amorphous sodium bisulphate.

TABLE 5

FECAL EXAMINATIONS FOR B. PARATYPHOSUS A

Number	Endo Positive	Dye-Agar Positive*	Percentage of Endo Positives Compared with Total Positives
856	30	60	50

* Majority of results based on use of one strength, 0.35-0.5 c c of a 0.1% solution of dye to 100 c c of agar.

TABLE 6

B. PARATYPHOSUS B

Number	Endo Positive	Dye-Agar Positive	Percentage of Endo Positives Compared with Total Positives
(11)*	5	11	45

* Encountered while examining for B. typhosus.

If, due to heavy seeding, diffusion occurs, typhoid colonies may nevertheless be identified on this medium by the characteristic translucent quality of the typhoid colony, which distinguishes it from the metallic sheen exhibited in most instances by even the smallest colony of the colon types. In case this distinctive appearance of the typhoid colony is not clearly perceived by daylight, this quality may be easily brought out by artificial illumination.

While this work was in progress, two plating mediums were reported: an eosin-methylene blue medium by Holt-Harris and Teague,[5]

[5] Jour. Infect. Dis., 1916, 18, p. 596.

and an eosin-brilliant green medium by Teague and Clurman.[6] The eosin-methylene blue medium depends on the application of the protective colloid principle. The 2 dye-solutions which in the concentrations used would mutually precipitate in aqueous solution, are prevented from combining when added separately to the agar, due to the protective action of the colloids in the medium. When fermentation occurs in the development of a colony, the protective action of the colloids is destroyed by the products of fermentation at this point, precipitation occurs, and the fermenting colony assumes a contrasting color.

The eosin-methylene blue medium has no differential restraining action on B. coli, and was suggested as a substitute for Endo's medium. We have worked with this medium and agree with the observations of Holt-Harris and Teague as to the sharp differentiation of the fermenting and nonfermenting types, the absence of any diffusion even with heavy seeding, and the lack of sensitiveness to the exposure of light. Since no diffusion occurs, a 1½% agar may be used as the basis of the medium instead of the 2½% agar usually required for Endo, which is a distinct advantage. Holt-Harris and Teague note one drawback to the eosin-methylene blue medium: the tendency of the reaction to disappear when growth is crowded, but claim that the medium will give successful differentiation, when an Endo plate equally inoculated would be unworkable on account of the diffusion of the indicator. We are not ready to agree with this contention. With a satisfactory Endo medium even an apparently hopeless plate can be fished by the aid of artificial illumination, the translucent, glassy typhoid colony being easily differentiated from the opaque, more deeply colored fermenting colonies which, even though small, nearly always show a distinctive metallic sheen. With diffusion on Endo, unless it is very intense, the search for the translucent typhoid colony seems to us no more difficult than on the equally deeply colored eosin-methylene blue agar. The colored background to our mind is the essential objection to the medium. In examining a large number of plates, it strains and tires the eyes rapidly, and obscures the differences between the typhoid colonies and other nonfermenting types, which are usually perceptible on Endo, to those accustomed to its use. Both mediums have advantages, and the selection must depend on personal preferences. For occasional or small series of examinations, the eosin-methylene blue medium would probably be preferable because of the stability of the

[6] Jour. Infect. Dis., 1916, 18, p. 647.

reagents used and the resulting greater uniformity of the product. We have found the extract agar we use for the brilliant green plating medium, to be a satisfactory basis for the eosin-methylene blue medium. Because of the transparent background and the tendency of the typhoid colony to take on positive characteristics, a properly standardized Endo plate gives the best picture obtainable, and because of these facts as well as the large number of examinations, we have continued its use in spite of its other drawbacks.

We have also tried the eosin-methylene blue medium with members of the paratyphoid and dysentery groups. With B. paratyphosus A and B, the results were as satisfactory as with B. typhosus. The dysentery types, particularly the Shiga, are apt to be irregularly restrained on this medium. Since these observations are based on stock cultures, they are only to be interpreted as a caution, as we have had no experience with freshly isolated fecal strains. We have noticed a similar tendency to irregular restraint, with dysentery strains on Robinson and Rettger's modification of Endo. Kendall's Endo has not shown this action if the plates were freshly poured.

The color change on the eosin-brilliant green medium of Teague and Clurman is less sharp than with the eosin-methylene blue medium. The essential claim made for the eosin-brilliant green medium was that the eosin acting probably by absorption reduces the activity of the brilliant green to a base level where it is no longer appreciably influenced by differences in the medium or by the fecal proteins inoculated. We have tried this medium to only a limited extent because of the difficulties encountered in obtaining what we considered a satisfactory end-product. These difficulties were in relation to obtaining suitable differential restraint and were probably due to the fact that a sufficient range of dilutions were not tried. In a later article, Teague and Clurman[7] suggest the preliminary use of several concentrations of brilliant green and that large batches of agar be prepared to limit the number of preliminary tests of the medium. In other words, the masking action of the eosin does not exclude the necessity of preliminary standardization and the determination of the optimum concentration of brilliant green in the routine examinations. There are other and more essential disadvantages to the medium. A meat-infusion agar is necessary. The comparatively deep color of the medium obscures any positive characteristics the typhoid colony may possess, eye-strain resulting from the attempt to differentiate the non-

[7] Jour. Med. Research, 1916, 35, p. 121.

lactose fermenting types. The eye-strain is enhanced by the peculiarly irritating color of the medium when viewed by transmitted light. This becomes a very serious drawback when the plates to be examined by one individual run into the hundreds. Both these difficulties are further enhanced when the plates become crowded as the differential color reaction tends to disappear. Since standardization is necessary, an almost colorless transparent medium which, however crowded, still retains the characteristic appearance of the typhoid colony seems to us preferable.

SUMMARY

The brilliant green medium we have devised has markedly increased the number of positive fecal examinations for B. typhosus and B. paratyphosus. The methods of preliminary and final identification previously recommended have been found reliable and have materially aided in making possible large numbers of examinations with the minimum of workers. The essential point in the success of the medium is proper standardization. No method has been devised to render such standardization unnecessary. The medium is most adapted to routine work where many specimens are examined, as in this case the time required for standardization is relatively slight, as large amounts of medium can be standardized and used before the results of the standardization tests are rendered void by the deterioration of the dye solution or evaporation of the medium. Of the recently suggested nonrestraining plating mediums, Robinson and Rettger's modification of the Endo agar has given a far superior contrast between lactose and nonlactose fermentation types than that obtainable with the various modifications thus far tried. The methylene blue-eosin medium of Holt-Harris and Teague gives a similarly striking contrast if the plates are not crowded, but has the objection of being a colored medium thus obscuring any differeneces between the colonies of the nonlactose types and tiring the eyes when numerous plates are to be examined. Both of these mediums tend to restrain the growth of some culture strains of B. dysenteriae, indicating that they may not be satisfactory for the isolation of this organism from feces.

THE THERMAL DEATH POINT AND LIMITING HYDROGEN ION CONCENTRATION OF PATHOGENIC STREPTOCOCCI

S. Henry Ayers, Wm. T. Johnson, Jr., and B. J. Davis

From the Research Laboratories of the Dairy Division, U. S. Department of Agriculture

Since the epidemic of septic sore throat in Boston in 1911 the thermal death point of streptococci has acquired an added importance, as that epidemic led to the belief that a streptococcus was the causative agent of the disease. Added responsibility was placed, therefore, on the process of pasteurization as a protective agent against septic sore throat.

Previous to the work of two of us[1] in 1910, it was a generally accepted fact that streptococci were destroyed at temperatures considerably lower than those commonly used in pasteurization. One strain of streptococcus was found the thermal death point of which was 75.6 C. (168 F.) when heated for 30 minutes, and many other streptococci which were able to resist a temperature of 65.6 C. (150 F.) for a period of 30 minutes. This gave a new aspect to the subject of the thermal death point of streptococci and led us[2] to further experiment along this line which showed that there was a wide variation in the thermal death point of 139 cultures of streptococci isolated from cow feces, milk and cream. When heated in milk under conditions similar to pasteurization at 60 C. (140 F.) the lowest pasteurizing temperature, 64.03% of cultures survived. At 62.8 C. (145 F.) the usual temperature for pasteurization, 33.07% of cultures survived. At 71.1 C. (160 F.) 2.58% survived and all were destroyed at 73.9 C. (165 F.). In all experiments the milk was heated, then held for 30 minutes. Davis[3] has also found that 20 of 74 strains of hemolytic streptococci from milk, and having practically no virulence, were destroyed when heated at 68.3 C. (155 F.) for 30 minutes.

Since it has been fairly definitely decided that the streptococci are the causative agents of septic sore throat the ability of certain of this

Received for publication April 8, 1918.

[1] Ayers, S. H., and Johnson, W. T., Jr.: U. S. Dept. Agri., Bur. Anim. Indust., 1910, Bull. 126.

[2] Ayers, S. H., and Johnson, W. T., Jr.: Jour. Agr. Research, 1914, 2, p. 321.

[3] Am. Jour. Pub. Health, 1918, 8, p. 40.

group of organisms to stand temperatures above that of pasteurization naturally presents a grave situation. If pathogenic streptococci are able to survive the usual process of pasteurization then the value of the process, from a sanitary standpoint, is materially lowered.

Experience with the use of properly pasteurized milk and the determination of the thermal death point of pathogenic streptococci by various investigators, indicate very clearly, however, that the thermal death point of these organisms is relatively low and that they are readily destroyed by proper pasteurization. Thus, Hamburger,[4] who studied the epidemic of septic sore throat in Baltimore in 1912, traced this epidemic to a certain milk supply. Advice was given to boil all milk and the dairy connected with the epidemic raised the temperature of their flash pasteurization to 71.1 C. (160 F.), then changed to the holder process where the milk was heated to 62.8 C. (145 F.) and held for a period of 30 minutes. The cases of sore throat that followed were neither so severe or so numerous and did not follow the milk supply but appeared to have been transmitted from individual to individual. He[5] also found that a streptococcus isolated from a patient having a case of sore throat was killed by heating in milk at 62.8 C. (145 F.) for 30 minutes. Again Capps and Miller,[6] who studied the Chicago epidemic of septic sore throat, traced it to a dairy where the milk was pasteurized by the flash process at 71.1 C. (160 F.). On certain dates they found that there was a pronounced failure to pasteurize and following these dates there were outbreaks of septic sore throat. These authors believed that the final responsibility for the epidemic rested on the inadequate and unreliable pasteurization. They state that the absolute protection of the children of the Michael Reese Hospital from infection by efficient pasteurization demonstrates this point. Bray,[7] who studied an epidemic of tonsillitis of tuberculous patients, traced the epidemic to a milk supply of one farm where a carrier presumably infected the milk. Forty cases of tonsillitis resulted among 400 people. As soon as the epidemic broke out the milk was pasteurized, and from that time only 1 case appeared.

From the results achieved from the proper pasteurization of milk it seems evident that the thermal death point of pathogenic streptococci, which cause septic sore throat, is relatively low. This belief is

[4] Jour. Am. Med. Assn., 1912, 58, p. 1109.
[5] Bull. Johns Hopkins Hosp., 1913, 24, p. 1.
[6] Jour. Am. Med. Assn., 1912, 58, p. 1848.
[7] Ibid., 1915, 64, p. 1127.

borne out by the results of the studies of Davis,[3] who found that streptococci isolated from cases of sore throat were readily killed by heating at 60 C. (140 F.) for 30 minutes. Davis[8] also found that none of 24 strains of pathogenic hemolytic streptococci of human origin resisted heating at 60 C. (140 F.) for 30 minutes. He makes the following statement: "I know of no evidence that strains of streptococci pathogenic to man can resist the usual temperature of pasteurization, 145 F., for 30 minutes."

In this paper it is desired to present further results bearing on the thermal death point of pathogenic streptococci. Thirty-five cultures of supposedly pathogenic streptococci were obtained from various sources. Some of these were sent to us in pure cultures after having been isolated from various cases of infection and others were isolated in our laboratory from pus and body fluids. All cultures were tested for pathogenicity by intravenous inoculation of rabbits. The hemolytic power was determined on blood-agar plates incubated for 24 hours. Fermentation tests were made and the thermal death point determined by inoculation of tubes of milk with several drops of fresh broth culture. These tubes were then heated at different temperatures for a period of 30 minutes in order to duplicate the process of pasteurization.

The results of this work are shown in Table 1. It will be noted from the table that all of the A series cultures were hemolytic. Many of them produced infections and often death in rabbits and all had a thermal death point at 60 C. (140 F.) or lower. In fact, only 5 showed a thermal death point as high as 60 C. (140 F.); 11 were destroyed at 57.2 C. (135 F); 6 at 54.5 C. (130 F.); and 5 at 51.7 C. (125 F.) out of a total of 27 cultures. Our results indicate, therefore, that pathogenic streptococci may be destroyed in milk by pasteurization at 60 C. (145 F.) for 30 minutes.

Further reference to the table shows 8 cultures in the B series, the source of which seemed to indicate that they might be pathogenic. Most of these cultures, however, were isolated from pus or body fluids, and it is quite possible that nonpathogenic streptococci were associated with the pathogenic strains and were obtained on plating instead of the more difficultly isolated pathogenic types.

It will be seen that none of the B series were pathogenic to animals. Six of the 8 cultures were not hemolytic and 1 of the other 2 only slightly so. Most of them had a high thermal death point. The

[8] Jour. Am. Med. Assn., 1912, 58, p. 1852.

most interesting point in connection with the B series of cultures was the difference in the limiting hydrogen ion concentration, in a dextrose-yeast-peptone medium. The P_H limit of the A series ranged from 5.4-6.0 and in the B series from 4.5-4.7.

TABLE 1

RESULTS OF A STUDY OF CULTURES FROM PATHOLOGIC SOURCES

No.	Source	Results of Rabbit Inoculation	Hemolysis	Dextrose Yeast-Peptone Broth P_H	Thermal Death Point °C.	Thermal Death Point °F.
A1	Puerperal infection	Joint enlargement, cultures recovered	+	5.7	60.0	140
A2	Puerperal infection	Death, cultures recovered	+	5.6	54.5	130
A3	Peritonitis	No effect	+	5.6	54.5	130
A4	Otitis	Partial paralysis hind legs.	+	6.0	57.2	135
A5	Tonsil	Lost	Lost	Lost	57.2	135
A6	Blood, meningitis	No effect	+ sl.	5.6	57.2	135
A7	Renal infection	No effect	+ sl.	5.6	57.2	135
A9	Septicemia, phlebitis	No effect	+	5.8	57.2	135
A11	Blood, death from gas bacillus infection	No effect	+ sl	5.5	57.2	135
A17	Cervical abscess	No effect	+ sl	5.5	60.0	140
A18	Necropsy, terminal infection by streptococcus septicemia	No effect	+ sl.	5.6	57.2	135
A19	Abscess in guinea-pig inoculated with B. mallei	Died, culture recovered	+	5.4	57.2	135
A22	S. pyogenes, Cornell Vet. College	Died, kidney and liver congested	+	5.6	54.5	130
A23	Sinus pus	No record	+ sl.	5.5	60.0	140
A24	Knee joint pus	No effect	+	5.8	57.2	135
A26	Knee joint fluid	No effect	+	5.5	60.0	140
A27	S. pyogenes (Rosenberger)	Died	+	6.0	51.7	125
A30	S. pyogenes (Holman)	Sick and recovered	+ sl.	5.5	54.5	130
A31	S. pyogenes (Homan)	Died	+	5.7	57.2	135
A32	Heart blood at necropsy	No effect	+ sl.	5.4	57.2	135
A33	Infected finger	No effect	......	5.5	51.7	125
A34	Infected finger	Sick, noduled in lungs when killed	+	5.4	51.7	125
A35	Pneumonia, guinea-pig	Died	+	5.5	57.2	135
A36	Peritonsillar abscess	Died	+	5.4	54.5	130
A37	Severe sore throat	Partially paralyzed	+	5.6	51.7	125
A38	Severe sore throat	Died	+	5.7	60.0	140
A39	Ear discharge, purulent otitis media	Paralysis of hind legs.	+	5.6	51.7	125
B13	Pleural pus	No effect	—	4.6	68.3	155
B14	Pleural fluid	No effect	+ sl.	4.6	65.6	150
B15	S. endocarditis, from blood	No effect	—	4.7	62.8	145
B20	S. wolfson (Steele)	No effect	—	4.6	62.8	145
B21	S. pyogenes X, Cornell Vet. College	No effect	+	4.6	60.0	140
B25	Elbow joint	No effect	Green	4.6	71.1	160
B28	Pleural pus	No effect	—	4.7	60.0	140
B29	Pus from scrotum	No effect	Green	4.6	71.1	160

The higher limiting hydrogen ion concentration with the B series cultures with the fact that none of them were pathogenic to rabbits, that most of them were not hemolytic, that they grew more vigorously in cultures, and in general they fermented a larger number of test

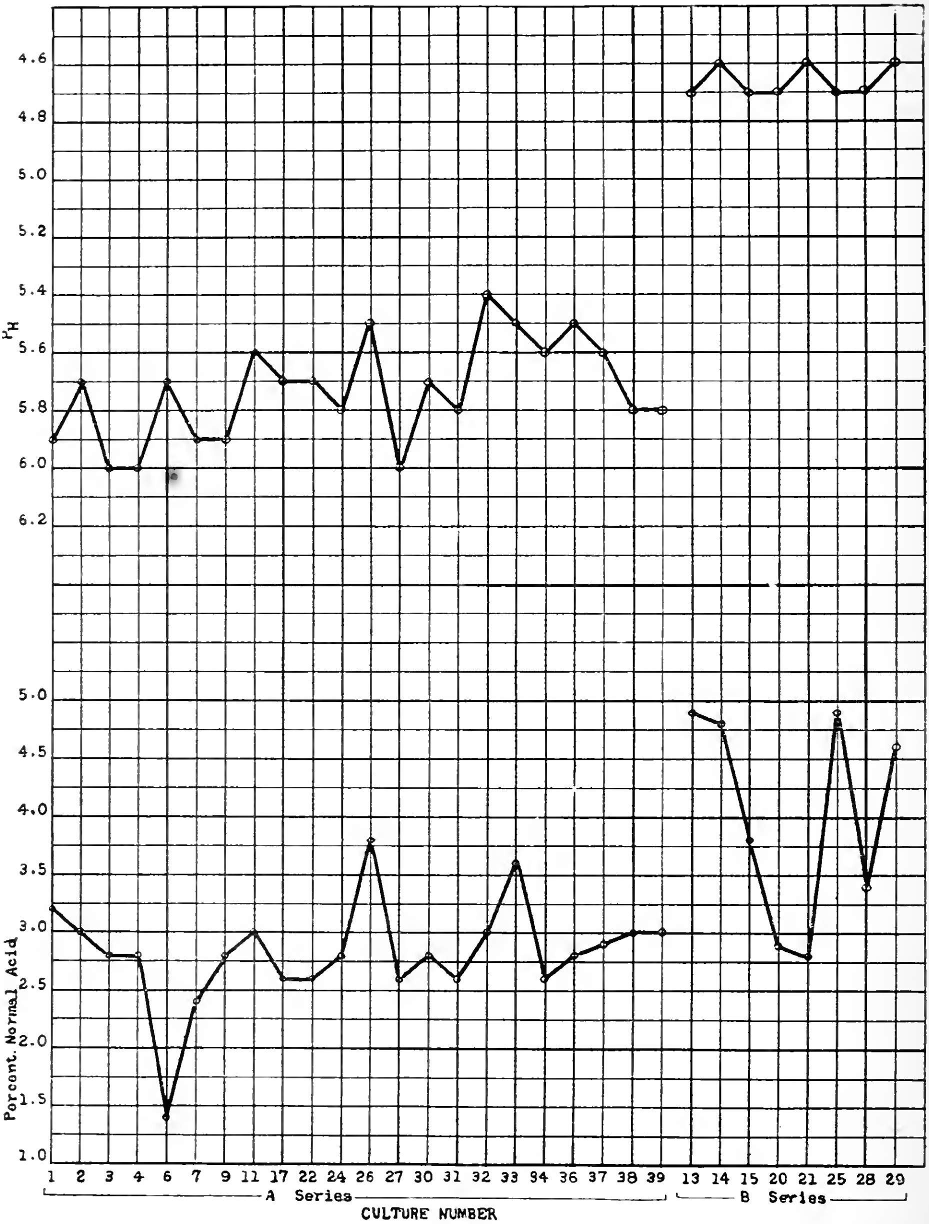


Chart 1.—Acidity by titration and the limiting hydrogen ion concentration of the A and B series.

substances than the A series, seems to separate them sharply from the pathogenic A series cultures. This leads to a further consideration of the limiting hydrogen ion concentration of pathogenic streptococci.

THE LIMITING HYDROGEN ION CONCENTRATION OF PATHOGENIC STREPTOCOCCI

Numerous investigators have found that in general the pathogenic streptococci produce less acid from carbohydrates and other test substances than the nonpathogenic strains. The differences in the amounts of acids formed, however, as shown by titration have not been sufficiently constant to clearly show a difference. Distinct differences in acid-forming ability are, however, clearly shown by means of the hydrogen ion concentration.

In Chart 1 is plotted the per cent of normal acid formed in a dextrose-yeast-peptone medium by cultures of the A and B series, together with the hydrogen ion concentration produced by the same cultures. This medium consisted of 1% dried yeast (a preparation known as cerevisine), 1% peptone and 1% dextrose, with the hydrogen ion concentration adjusted to P_H 7.2.

From the graph it is apparent that titration did not show a constant difference in the amount of acid formed by the A and B series of cultures. While in general the B series show more acid, 4 of the 8 cultures produced a quantity of acid which was within the range of the amounts formed in the A series. A glance at the curves, in the upper part of Chart 1 of the hydrogen ion concentration reached in the same medium, shows a distinct difference in concentration between the A and B series. The P_H values reached by the A series were from 5.4-6.0 while those of the B series were from 4.6-4.7. It is considered that these ranges in P_H values are the limiting hydrogen ion concentration only for the medium used in our experiments. These limits of hydrogen ion concentration might possibly be changed in other mediums with different concentrations of sugar and different buffer actions.

It is believed that the difference in the limiting hydrogen ion concentration between the A series, which is considered to be the pathogenic streptococci, and the B series, which is considered to be the nonpathogenic, represents a distinct differential characteristic. This opinion is strengthened by the figures in Table 2, where it will be seen that of 34 cultures isolated from pathologic sources the

limiting hydrogen ion concentration of 26, or 76.5%, ranged from P_H 5.4-6.0 while 8, or 23.5%, showed limiting P_H values from 4.5-5.0.

These results should be compared with cultures obtained from human mouths, from feces, udders and mouths of cows, and milk. Of the 18 cultures from the human mouth all showed a high limiting hydrogen ion concentration. Among the cultures obtained from feces, udders and mouths of cows 82 cultures out of a total of 86, or 95.4%, showed a high limiting hydrogen ion concentration. Among the 60 cultures isolated from milk, 54, or 90%, showed a high limiting hydrogen ion concentration ranging from 4.5-5.0, while 6, or 10%, ranged from 5.4-6.0. The 6 cultures while showing the same limiting hydrogen ion concentrations as the A Series, considered pathogenic strepto-

TABLE 2

STREPTOCOCCI GROUPED ACCORDING TO SOURCE AND LIMITING HYDROGEN ION CONCENTRATION

P_H	Pathologic Sources	Human Mouth	Feces, Udders and Mouths of Cows	Milk
4.5-5.0	Hemolysis slight 2 negative 6 8-23.5% B series	Hemolysis — 18-100%	82-95.4%	Hemolysis — 54-90%
5.4-6.0	Hemolysis + 26-76.5% A series	Hemolysis — 0-0%	4-4.6%	Hemolysis — 6-10%
Total	34	18	86	60

cocci, differed considerably in that they were not hemolytic and in general fermented a much larger number of carbohydrates and other test substances. The limiting hydrogen ion concentrations were obtained in dextrose-yeast-peptone cultures and it is interesting to mention that the same limiting hydrogen ion concentrations were reached in all of the carbohydrates and other test substances, which the organisms were capable of fermenting.

The difference in the limiting hydrogen ion concentration among the streptococci has been previously noted.[9] More recently, Smillie[10] found that as a general rule the more pathogenic streptococci produced a lower acidity than the less pathogenic strains. Most of his acidity determinations were made by means of titrations but he determined the hydrogen ion concentration of 5 cultures. The liberty is taken of reproducing a table in revised form from his paper which may

[9] Ayers, S. H.: Jour. Bacteriol., 1916, I, p. 84.
[10] Jour. Infect. Dis., 1917, 20, p. 45.

be seen in Table 3. This shows the limiting hydrogen ion concentrations reached by 5 cultures, one of which was evidently nonpathogenic. It is of interest to note that the nonpathogenic strain, No. 57, reached a limiting hydrogen ion concentration P_H 4.5, while that of the pathogenic strains averaged around P_H 5.5.

In a study of the sugar fermentation Smillie used sugar-free veal infusion broth as a basic medium and it is rather interesting that he obtained two limiting hydrogen ion concentratons, which agree with those obtained by us in a dextrose-yeast-peptone medium.

Although Smillie did not give any particular consideration to the significance of these limiting hydrogen ion concentrations his results,

TABLE 3

LIMITING HYDROGEN ION CONCENTRATION OF STREPTOCOCCI AS GIVEN BY SMILLIE

No.	Type of Hemolysis	Virulence for Animals 24-Hour Culture	Sugar Reactions PH*							
			Dextrose	Lactose	Maltose	Saccharose	Mannite	Salicin	Raffinose	Inulin
57	Nontypical beta incomplete	0.5 c c did not kill mouse	4.5	4.4	4.6	4.4	7.4	4.7	4.3	4.7
56	Beta	0.5 c c killed mouse in 7 days	5.1	5.5	5.4	5.5	7.2	5.7	7.0	7.2
58	Beta	0.5 c c killed mouse in 6 days	5.4	5.5	5.6	5.6	7.2	5.5	7.2	7.0
59	Beta	0.5 c c killed mouse in 3 days	5.7	5.5	5.6	5.8	7.2	5.7	7.2	7.2
50	Beta	0.5 c c killed mouse in 2 days	5.4	5.5	5.3	5.4	7.0	5.6	7.0	7.0

* Control PH 7.2.

with even a few cultures, strengthen our opinion that the limiting hydrogen ion concentration of streptococci will prove of value and it at least deserves further careful study.

The value of the determination of hydrogen ion concentration is clearly shown by the results previously discussed and in this connection it is desired to emphasize another point, which is, that the determination of the hydrogen ion concentration gives the limiting value reached in the fermentation regardless of the point at which it starts, provided of course, it is within the growing range of the organism. On the other hand, with titration methods the original reaction may be so high that when the control titration is subtracted from the total titration the difference will be less than 1% normal acid, an amount which is usually considered the lowest limit for showing fermenta-

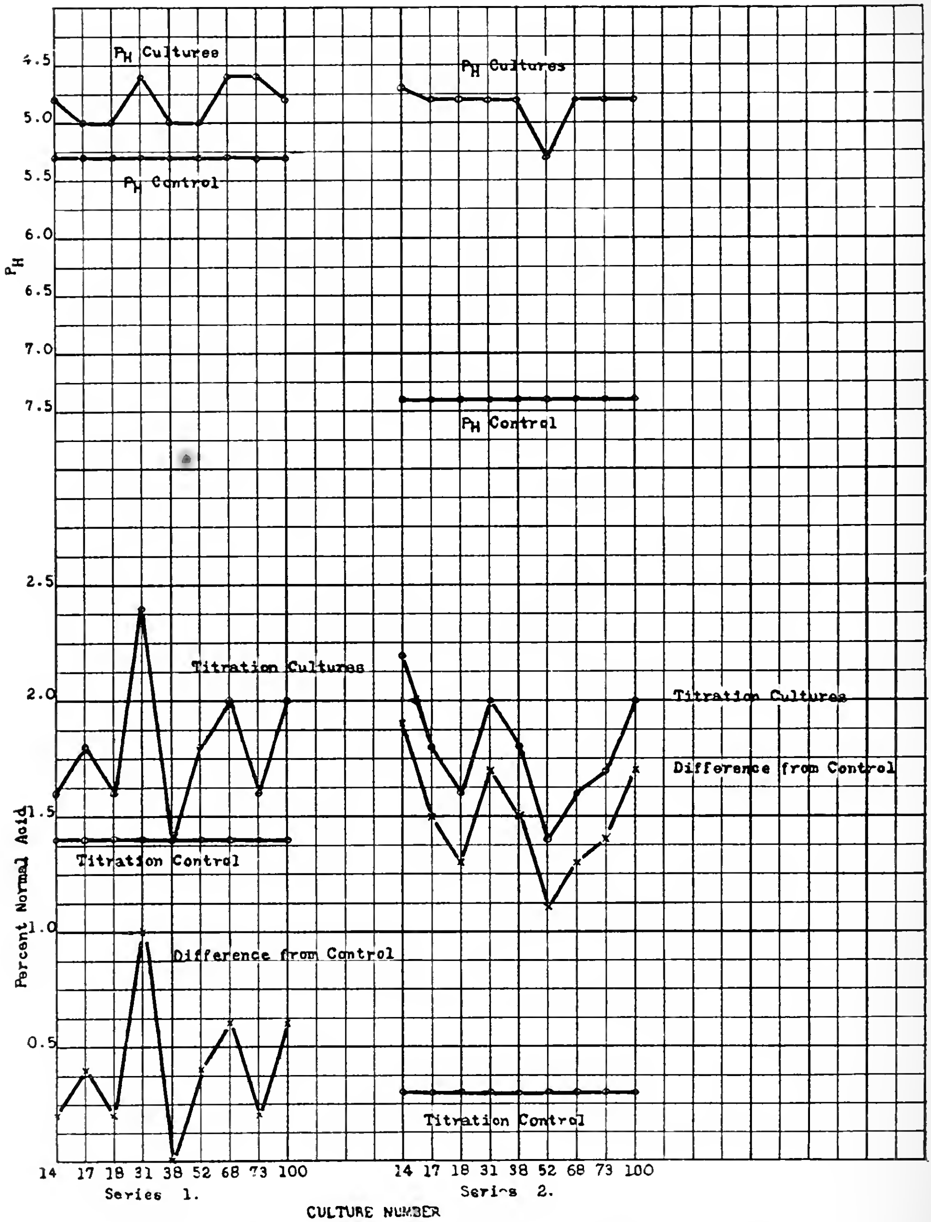


Chart 2.—Acidity as measured by titration and hydrogen ion concentration.

tion. This point is very clearly shown in Chart 2. A series of 9 cultures were inoculated into a dextrose-yeast-peptone medium and the reaction corrected to plus 1.4 (Fuller's scale) and the same cultures were inoculated into the same kind of broth having a reaction of plus 0.3 (Fuller's scale). After incubation for 7 days the cultures were titrated. The results are plotted at the bottom of Chart 2 where the curves represent the total titration, titration of the control, and the difference between the control and the total titration. On the left hand side of Chart 2 it will be observed that when the control titration of plus 1.4 was subtracted from the total titration the difference of per cent of normal acid formed was never above 1%. If, therefore, 1% of acid or less was considered negative fermentation then it would be said that none of the 9 cultures fermented dextrose. When the original reaction was plus 0.3 (Fuller's scale) and the control titration subtracted from the total titration and plotted as shown on the right hand side of Chart 2 it will be seen that the per cent. of acid formed in the same set of cultures was always above 1% acid. It would be considered, therefore, that these cultures fermented dextrose.

Based on titration methods there is a condition where with a high initial reaction the fermentation tests would be considered negative while with a low initial reaction they would be considered positive. When the series of cultures were inoculated into the medium of high and low reaction according to Fuller's scale duplicate sets were also inoculated and hydrogen ion concentration determined after 7 days' incubation. Results plotted at top of Chart 2 show that in the medium having an initial reaction of plus 1.4 (Fuller's scale) the hydrogen ion concentration of the control tubes was 5.3 and the cultures after incubation showed a limiting hydrogen ion concentration of from 4.6-5. In the other series where the original reaction was plus 0.3 the hydrogen ion concentration of the control tubes was 7.4 and after incubation it ranged from 4.7-5.4. It is apparent from this, therefore, that while by titration methods the cultures starting with a high reaction, that is plus 1.4 (Fuller's scale), would be said not to ferment dextrose, the hydrogen ion determinations would show that they fermented and reached their limiting hydrogen ion concentration the same as when their original reaction was at plus 0.3. In other words, the determination of hydrogen ion concentration would show a fermentation in both sets of mediums.

This point has been emphasized more perhaps than would at first seem warranted, but it is points of this nature that must be most carefully observed in the determination of the fermenting ability of bacteria.

SUMMARY AND CONCLUSIONS

The thermal death point of 27 strains of pathogenic streptococci from pathologic sources was never higher than 60 C. (140 F.) when heated in milk for 30 minutes. This fact, together with the results obtained by other investigators, seems to indicate that pathogenic streptococci are destroyed by the proper pasteurization of milk at 60 C. (140 F.) for a period of 30 minutes.

Some of eight cultures isolated from pathologic sources had thermal death point above 60 C. (140 F.) for 30 minutes and would, therefore, survive pasteurization. From their cultural characteristics we believe that these cultures were not pathogenic strains.

Our results indicate that in a dextrose-yeast-peptone medium, as used in our experiments, pathogenic streptococci reach a limiting hydrogen ion concentration from 5.4-6, while a nonpathogenic strain reached a higher concentration, namely, P_H 4.5-4.9. It is believed that this difference in limiting hydrogen ion concentration together with other characteristics such as the hemolytic power of the organism and the fermentation of carbohydrates and other test substances may serve as an important differential characteristic between pathogenic and nonpathogenic streptococci. Our results on this point are not at all conclusive and need confirmation by a study of a large number of cultures.

The value of the determination of hydrogen ion concentration in the study of fermentation cannot be too strongly emphasized and it is believed that titration methods for the determination of the fermentation of test substances should be discarded.

A STUDY OF THE NEUTRALIZING PROPERTIES OF ANTIPOLIOMYELITIC HORSE SERUM

(NEUTRALIZATION OF VIRUS IN VITRO)

John W. Nuzum

From the Pathologic Laboratory of the Cook County Hospital, Chicago

The observation that a pleomorphic coccus is present in the central nervous system in poliomyelitis in man as well as in the experimental disease in monkeys, has been confirmed generally and apparently no longer is open to any serious question.

In contrast with the globoid bodies of Flexner and Noguchi,[1] which possess but feeble antigenic properties, it has been shown that intravenous injections of the poliomyelitic coccus in animals produce antibodies in high concentration[2] and that such serum possesses protective and curative properties[3] in experimental poliomyelitis of monkeys. The application of this knowledge to the treatment of poliomyelitis in man was made by Nuzum[4] who treated 10 cases of poliomyelitis with promising results during the latter part of the Chicago epidemic of 1916. Subsequently a total of 220 patients were treated with immune horse serum during the Chicago epidemic of 1917 and the favorable results[5] appear to coincide with those obtained by Rosenow[6] in a series of 58 cases occurring in Davenport, Iowa.

The apparently beneficial results from serum therapy, especially when used early in the preparalytic stage of the disease, and the harmlessness of the method when all due precautions are observed, justify a thorough and extensive trial of the serum. At the same time it is of the greatest importance to use only serum which possesses specific antibodies, if good results are to be obtained.

It is well known that recovery from poliomyelitis is accompanied by the appearance of neutralizing substances in the blood serum; this

Received for publication April 10, 1918.

1 Jour. Exper. Med., 1917, 25, p. 545.

2 Nuzum, J. W.: Jour. Am. Med. Assn., 1917, 68, p. 24.

3 Rosenow, E. C.: Jour. Am. Med. Assn., 1917, 69, p. 261. Nuzum, J. W.: Jour. Infect. Dis., 1918, 22, p. 258.

4 Bull. Chicago Med. Soc., April 21, 1917.

5 Nuzum, J. W., and Willy, R. G.: Jour. Am. Med. Assn., 1917, 69, p. 1247.

6 Jour. Infect. Dis., 1918, 22, p. 379.

is the case in both man and monkey. The test of the potency of antipoliomyelitic horse serum, I believe, can be accurately determined in monkeys by the neutralizing properties of the serum against active virus in vitro. A study of this neutralization of virulent monkey virus by serum in vitro forms the basis of this paper.

EXPERIMENTS

A highly active monkey adapted virus was employed, which is now in its 25th generation and possesses the maximal degree of virulence for monkeys. I have followed the technic used by others in this field. A 5% suspension of fresh or recently glycerolated spinal cord and brain in normal salt solution was carefully prepared by grinding the tissues in a mortar with sterile sand and filtering 3 times through coarse filter paper to remove any gross particles of brain tissue. In other instances the suspension was passed through a Berkefeld filter and the clear filtrate employed in the tests. The immune horse serum was thoroughly mixed with the filtrate; for controls, similar mixtures of filtrate with normal horse serum and immune serum were used; the mixtures were placed in the incubator for 3 hours and in the ice-chest for 18-21 hours prior to inoculation into monkeys. In each series of experiments rhesus monkeys of approximately equal size were chosen. Each animal received the respective mixture of serum and virus intracerebrally under ether anesthesia. The protocols follow:

SERIES 1.

Exper. 1.—Monkey 63. (Control.) Dec. 5, 1917, 3 p. m.: Monkey etherized and given an intracerebral injection of 0.5 c c of a 5% suspension of fresh cord and brain of paralyzed Monkey 60.

Dec. 11: Onset of symptoms with marked tremor of the head. Climbs awkwardly in the cage.

Dec. 12: Flaccid paralysis of both legs.

Dec. 13: Severely paralyzed in all extremities.

Dec. 14: Prostrate in cage and in a dying condition. Typical lesions of poliomyelitis.

Exper. 2.—Monkey 64. (Control.) Dec. 5, 1917, 3:30 p. m.: Etherized and given an intracerebral injection of 0.3 c c of a 5% suspension of fresh Monkey Virus 60.

Dec. 12: Tremor of head and neck muscles. Animal quiet in cage.

Dec. 13: Complete flaccid paralysis of the legs. Marked tremor.

Dec. 14: Paralysis extending. Arms weak. Cannot sit up in the cage. This monkey survived the infection and ultimately regained good use of the arms. The hind legs remained flaccid and now exhibit marked atrophy and contractures.

Exper. 3.—Monkey 65. Dec. 5, 1917: 0.3 c c of Virus 60 plus 0.6 c c of immune horse serum were thoroughly mixed, placed in the thermostat for 3 hours and then in the ice-box for 21 hours. Monkey was etherized and given an intracerebral injection of this virus serum mixture.

Dec. 20: Monkey remains well and apparently normal.

Jan. 10: Has never exhibited any symptoms of poliomyelitis.

Exper. 4.—Monkey 66. (Control.) Dec. 5, 1917: 0.5 c c of virus plus 0.5 c c of normal horse serum were previously thoroughly mixed and incubated for 3 hours and placed in the ice-box for 21 hours. Monkey etherized and injected intracerebrally with this mixture.

Dec. 10: Monkey prostrate in cage. 10 p. m.: marked respiratory embarrassment. Death. Microscopic sections of the medulla and cervical cord revealed marked lesions of poliomyelitis.

Exper. 5.—Monkey 67. Dec. 5, 1917: Intracerebral injection of a virus serum mixture prepared as follows: 0.5 c c of Virus 60 plus 0.5 c c immune horse serum were mixed and incubated for 3 hours and placed in the ice-box for 21 hours.

Dec. 20: Monkey has remained well.

Dec. 26, 12 a. m.: Monkey found prostrate in cage with flaccid paralysis of legs and weakness of right arm.

Dec. 28: Severely paralyzed in all extremities. Death at 7 p. m. Typical cord lesions of poliomyelitis.

Exper. 6.—Monkey 68. Dec. 5, 1917: Etherized and given an intracerebral injection of a virus serum mixture prepared as follows: 0.5 c c of Virus 60 plus 0.5 c c fresh immune human serum were mixed in the test tube and placed in the thermostat for 3 hours and then in the ice-box for 21 hours.

Dec. 21, 9 a. m.—Monkey has a flaccid paralysis of both legs.

Dec. 23: Prostrate in cage.

Dec. 25: Died. Microscopic sections of the medulla and cord reveal typical lesions of poliomyelitis.

In the experiments in Series 1 the inactivated immune horse serum was obtained Nov. 11; preserved with 0.1% tricresol, passed through a Berkefeld filter and stored in the ice-chest 24 days prior to use. Three control monkeys, receiving virus alone or virus plus normal horse serum (Monkey 66), all developed poliomyelitis within periods of 5-7 days subsequent to inoculation. Monkey 65 was completely protected against the disease by the immune horse serum.

Monkeys 67 and 68 inoculated with virus plus immune horse serum and virus plus immune human serum exhibited a definite delay of 16 and 11 days, respectively, in the onset of the paralysis over that of the control Monkey 66. This observation is of especial interest since we have here a parallel between the antibody content of immune horse serum and the serum of a recently recovered human which is known to possess neutralizing properties against virus when the proper experimental conditions are fulfilled.

SERIES 2

In this series of tests the immune horse serum was obtained Jan. 8 and used fresh without preservative. All monkeys were injected with fresh 5% emulsion of cord and brain of paralyzed Monkey 18. The experiments were controlled both with virus alone and also with virus plus immune human serum known to possess neutralizing properties.

Exper. 7.—Monkey 69. (Control.) Jan. 9, 1918, 7 p. m.: Etherized and given an intracerebral injection of 0.2 c c of a 5% suspension of the cord from paralyzed Monkey 18.

Jan. 14: Onset of symptoms, namely, monkey huddled in corner of cage. Grinds teeth. Fine muscular twitchings.

Jan. 27: Return of previous symptoms. Monkey very excitable. Marked tremor. Weakness of left arm.

Jan. 28: Both legs and left arm flaccid. Monkey prostrate in cage.

Jan. 30: Died. Sections of medulla, cervical and lumbar cord revealed typical lesions of poliomyelitis.

Exper. 8.—Monkey 70. Jan. 9, 1918: Etherized and intracerebral injection of a virus serum mixture prepared as follows: 0.4 c c of Virus 18 plus 0.6 c c immune horse serum were carefully mixed and incubated for 2 hours and placed in the ice-box for 22 hours.

Feb. 15: Monkey remains well. Has never shown any symptoms of poliomyelitis.

Exper. 9.—Monkey 71. Jan. 9, 1918: Etherized and given an intracerebral injection of virus serum mixture prepared as follows: 0.3 c c of virus plus 0.3 c c immune horse serum were thoroughly mixed and incubated for 2 hours and placed in ice-chest for 22 hours.

Feb. 15: Monkey has remained entirely normal.

Exper. 10.—Monkey 72. Jan. 9, 1918: Intracerebral injection of a virus serum mixture composed of 0.5 c c of virus plus 1 c c immune horse serum previously incubated as previously described.

Feb. 15: Monkey has never exhibited any symptoms of poliomyelitis. Remains well.

Exper. 11.—Monkey 73. Jan. 9, 1918: Intracerebral injection of virus serum mixture prepared as follows: 0.3 c c of virus plus 0.6 c c immune horse serum were thoroughly mixed in test tube and incubated 2 hours and placed in ice-chest for 22 hours.

Feb. 15: Monkey remains well.

Exper. 12.—Monkey 74. (Control.) Jan. 9, 1918: Intracerebral injection of a virus serum mixture prepared as follows: 0.3 c c of virus plus 0.6 c c immune human serum were mixed and incubated 2 hours and placed in the ice-chest 22 hours before injection.

Feb. 15: Monkey remains entirely normal.

Exper. 13.—Monkey 75. (Control.) Jan. 9, 1918: Intracerebral injection of virus serum mixture prepared as follows: 0.4 c c of virus plus 1 c c immune human serum were mixed in a test tube and incubated 2 hours and placed in the ice-box 22 hours prior to injection.

Feb. 15: Perfect neutralization of the virus. The monkey remains well.

Exper. 14.—Monkey 76. Jan. 9, 1918: 0.4 c c of virus plus 1 c c of immune horse serum were thoroughly mixed, placed in the thermostat and in the ice-chest the usual time and injected intracerebrally under ether anesthesia into Monkey 76.

Feb 15: Monkey has remained well and has never shown any symptoms of poliomyelitis.

An analysis of this series of eight inoculations shows that the 5 animals receiving virus plus immune horse serum were completely protected against infection, even when 2½ fatal doses of virus were employed. Control Monkeys 74 and 75 inoculated with virus plus serum from convalescent poliomyelitic patients remain well, showing the technic of the experiments to be adequate for the neutralization of virus by serums known to possess such action. And finally Monkey 69 receiving but 0.2 c c virus developed typical poliomyelitis as demonstrated microscopically thereby proving the activity of the virus.

SERIES 3

For this series of inoculations all serums were reactivated with fresh guinea-pig complement in the proportion of 1 part of complement to 9 parts of serum. A 5% emulsion of recently glycerolated monkey viruses (Nos. 64 and 68) was prepared, filtered 3 times through coarse paper and employed in all these tests. The virus serum mixtures were in every instance thoroughly mixed and placed in the thermostat for 3 hours and in the ice-chest 21 hours prior to injection.

Exper. 15.—Monkey 77. (Control.) Jan. 19, 1918, 3 p. m.: Intracerebral injection of 0.5 c c of 5% virus suspension.

Jan. 23: Onset of symptoms, namely, excitability and tremor of head.

Jan. 25: Flaccid paralysis of both legs. Marked generalized muscular twitchings. Highly excitable.

Feb. 25: Monkey survived with a complete flaccid paralysis of the hind legs. Marked atrophy of legs.

Exper. 16.—Monkey 78. Jan. 19, 1918: Intracerebral injection of a virus serum mixture prepared as follows: 0.5 c c of above virus was mixed with 0.5 c c immune horse serum and incubated for 3 hours and placed in the ice-box for 24 hours before inoculation.

Feb. 25: Monkey has remained perfectly well.

Exper. 17.—Monkey 79. Jan. 19, 1918: 1 c c of immune horse serum was thoroughly mixed with 0.5 c c virus, incubated 3 hours and placed in ice-box over night. This mixture was injected intracerebrally under ether anesthesia.

Feb. 25: The monkey remains well.

Exper. 18.—Monkey 80. Jan. 19, 1918: A virus serum mixture consisting of 0.75 c c virus plus 1 c c immune horse serum was prepared and after the usual incubation period, the above mixture was injected intracerebrally.

Feb. 25: Monkey has never shown any symptoms of poliomyelitis.

Exper. 19.—Monkey 81. (Control.) Jan. 19, 1918: A large rhesus received an intracerebral injection of a virus serum mixture as follows: 1 c c of normal horse serum was thoroughly mixed with 1 c c of virus, reactivated with 0.2 c c guinea-pig complement and placed in the thermostat for 3 hours and in the ice-box 21 hours prior to injection.

Jan. 23: Monkey had a convulsion. Fur ruffled. Muscular tremor.

Jan. 25: Repeated convulsions. Legs flaccid. Arms spastic.

Jan. 26: Monkey died during a convulsion. Microscopically typical lesions of poliomyelitis in the medulla, pons and cord.

Exper. 20.—Monkey 82. Jan. 19, 1918: 1 c c of virus suspension was mixed with 1 c c immune horse serum, incubated 3 hours and placed in ice-chest 21 hours. The above virus serum mixture was then injected intracerebrally under ether anesthesia.

Feb. 25: Monkey has never shown the slightest symptoms of poliomyelitis and remains well.

Exper. 21.—Monkey 83. (Control.) Jan. 19, 1918: 1 c c of virus suspension was mixed with 1 c c of immune human serum, incubated 3 hours and allowed to stand in the ice-box over night. This mixture was injected intracerebrally into Monkey 83.

The four monkeys receiving immune horse serum plus virus were completely protected against infection. Normal horse serum does not possess this neutralizing power against virus as shown in the case of Monkey 81. Monkeys 77 and 83 serve to control these experiments both positively and negatively.

SERIES 4

In this last series of experiments an attempt was made to determine the maximal dose of virulent virus which can be neutralized by immune horse serum. A fresh 5% Berkefeld filtrate of the spinal cord of a prostrate paralyzed monkey was carefully prepared. The serum employed was obtained fresh the same day. The protocols follow:

Feb. 25: Perfect neutralization. The monkey remains entirely normal.

Exper. 22.—Monkey 84. (Control.) Feb. 9, 1918, 10 a. m.: Etherized and given an intracerebral injection of 0.5 c c of a 5% suspension of fresh virus.

Feb. 14: Onset of symptoms. Excitable.

Feb. 16: Monkey completely paralyzed in all extremities. Cannot raise head up from floor of cage.

Feb. 19: Etherized in dying condition. Marked typical lesions of poliomyelitis in the medulla, pons, cervical and lumbar levels of the spinal cord.

Exper. 23.—Monkey 85. Feb. 9, 1918: Intracerebral injection of a virus serum mixture prepared as follows: 1 c c of 5% emulsion of virus was thoroughly mixed with 1 c c of fresh immune horse serum and incubated for 3 hours and placed in the ice-box for 21 hours before injection.

March 20: Monkey remains well and has never had any symptoms of poliomyelitis.

Exper. 24.—Monkey 86. Feb. 9, 1918, 12 noon: 2 c c of a fresh 5% suspension of virulent virus was carefully mixed with 2 c c of fresh immune horse serum and placed in the thermostat for 3 hours and in the ice-box over night. This entire virus serum mixture totaling 4 c c was injected half into each cerebral hemisphere under ether anesthesia.

Feb. 14: Monkey appears excitable and races about his cage.

Feb. 15: Tremor of head and neck.

Feb. 16: Flaccid paralysis of both legs. Arms weak. Ptosis of right upper eyelid.

Feb. 28: Monkey has survived although so completely paralyzed as not to be able to raise his head from the floor.

March 20: Monkey still living with marked atrophy of all muscles of the trunk and extremities and beginning contractures.

It thus appears that immune horse serum neutralized 1 c c of a 5% suspension of virulent virus perfectly but could not neutralize 2 c c of this virus. That the virus was attenuated somewhat seems plausible since this monkey survived although so severely paralyzed as to be unable to take food alone.

DISCUSSION

A series of 24 monkeys were inoculated intracerebrally with virulent monkey adapted virus plus various serums after previous incubation of the virus serum mixtures for 2-3 hour periods followed by storage in the ice-box for 21 hours. Under the conditions of the experiment, immune human serum neutralized virus perfectly in 3 monkeys.

Immune horse serum protected 11 monkeys perfectly against the virus of poliomyelitis. In the 2 monkeys in which both immune human serum and immune horse serum failed to neutralize the virus, it appears noteworthy that the immune horse serum caused a delay of 16 days in the onset of paralysis as compared with a delay of but 11 days with the serum of a convalescent poliomyelitic patient which is known to possess neutralizing properties against the virus of this disease.

Fresh virulent monkey adapted virus of maximal potency was utilized in these experiments, recently glycerolated virus was employed in a few instances. The immune horse serum has been used both fresh and after storage for periods of 24 days in the ice-box, both with and without preservative and both activated and inactivated. The experiments were carefully controlled both positively and negatively. The 5 control monkeys receiving virus alone all developed typical symptoms of poliomyelitis even with virus doses of 0.2 c c of a 5% suspension thereby indicating the virulence of the virus employed.

The 2 monkeys receiving normal horse serum plus virus developed a typical, rapidly fatal poliomyelitis as proven microscopically. This is in accord with previous observations made by myself and others, i. e., normal horse serum does not possess the power to neutralize suitable doses of virulent virus but rather tends to shorten the incubation period of the disease. Finally the neutralizing properties of immune horse serum as demonstrated by these experiments would appear to justify the use of such a serum in the treatment of poliomyelitis in man.

SUMMARY

Antipoliomyelitic horse serum, prepared by repeated intravenous injections of the coccus isolated from the central nervous system in human and monkey poliomyelitis possesses neutralizing properties against the virus of poliomyelitis.

Immune horse serum protected 11 monkeys perfectly against fatal doses of virus. In 2 monkeys in which both immune human serum and immune horse serum failed to neutralize the virus, a definite delay of 16 days in the onset of paralysis must be attributed to the immune horse serum as compared to a shorter delay of 11 days with immune human serum known to possess much neutralizing principles.

The neutralizing properties of antipoliomyelitic horse serum in vitro for the virus of poliomyelitis afford a convenient and satisfactory test of the potency of this serum for use in the treatmnt of poliomyelitis. Suitable controls with normal horse serum and comparsions with immune human serums known to possess neutralizing properties should be conducted simultaneously.

Finally, the neutralizing, protective and curative properties of antipoliomyelitic horse serum for experimental poliomyelitis of monkeys are in direct accord with the favorable results observed in the serum treatment of human poliomyelitis and argue strongly for the etiologic relationship of the coccus to this disease.

THE PROTECTIVE PROPERTIES OF ANTI-POLIOMYELITIC HORSE SERUM

John W. Nuzum

From the Pathologic Laboratory of the Cook County Hospital, Chicago

In previous experiments in a series of 53 monkeys we have demonstrated the protective, curative[1] and neutralizing[2] properties of anti-poliomyelitic horse serum for experimental poliomyelitis of monkeys. The striking beneficial results observed by Nuzum[3] and by Rosenow[4] in the treatment of human poliomyelitis with immune serum prepared by injections of the poliomyelitic coccus in animals demand a thorough and extensive trial of the serum.

It is obvious that to obtain the best results some convenient and satisfactory test of the potency of immune horse serum must be adopted to determine its fitness for use in human poliomyelitis. I have shown that such a test[2] may be made by an accurate estimation of the neutralizing properties of immune horse serum in vitro for the virus as determined by monkey inoculations.

Such a test, when properly controlled with normal horse serum, and also with immune serum obtained from both convalescent patients and monkeys which is known to possess neutralizing properties for virus, affords accurate information.

Recently, Amoss and Eberson[5] devised a therapeutic protective test to which he subjected a sample of Rosenow's serum. Two monkeys treated by intraspinal injections of the serum developed poliomyelitis. They conclude that the Rosenow serum is devoid of protective power.

With the purpose of determining the value and limitations of such a protective test, and in addition, to determine the protective value of our serum, I have made this test in 3 different series of monkeys.

The test rests primarily on the observation of Flexner and Amoss that intraspinal injections of normal horse serum or monkey serum set up an aseptic inflammation of the meninges and choroid plexus

Received for publication April 10, 1918.

[1] Nuzum, J. W., and Willy, R. G.: Jour. Infect. Dis., 1918, 22, p. 258.
[2] Nuzum, J. W.: Jour. Infect. Dis., 1918, 23, p. 301.
[3] Bull. Chicago Med. Soc., April 21, 1917; Jour. Am. Med. Assn., 1917, 269, p. 1247.
[4] Jour. Infect. Dis., 1918, 22, p. 379.
[5] Jour. Exper. Med., 1918, 27, p. 309.

in monkeys thereby facilitating the passage of virus from the blood stream into the nervous tissues and thus inducing characteristic lesions and symptoms of poliomyelitis. They found that only one substance possessed the power, when injected intraspinally, to prevent the localization of the virus in the nervous system, namely, the serum of convalescent monkeys which have survived a poliomyelitic infection.

The actual test is carried out as follows: An active, fresh virus is obtained by inoculating a monkey intracerebrally with glycerolated virus in the usual manner. By virus, I mean 5% emulsions of the brain or spinal cord of paralyzed monkeys and not cultures of the poliomyelitic coccus. The virus utilized in all experiments recorded in this manuscript was originally obtained from Dr. N. E. Wayson, of the Public Health Service, Washington, D. C. It has now been propagated through a series of 25 monkeys and has reached the maximal degree of potency for all monkeys not immune. When prostrate, the monkey is etherized and the spinal cord and medulla removed aseptically and a 5% suspension of the nervous tissue in normal salt solution prepared, centrifuged and 50 c c of the clear supernatent fluid injected intravenously into monkeys. Control animals and monkeys treated with the serum to be tested, normal horse serum and convalescent monkey serum are injected similarly and tested at the same time. Daily intraspinal injections of the serums to be tested are made each day for 4 successive days. Following a rest of 2 days 3 additional intraspinal injections are given.

SERIES 1

Exper. 1.—Monkey 87. (Control.) Feb. 7, 1918: Intravenous injection of 50 c c of the fresh prepared 5% suspension of monkey virus which was previously passed through a Berkefeld filter.

March 22: Monkey has remained well.

Exper. 2.—Monkey 88. (Normal horse serum control.) Feb. 7, 1918, 3 p. m.: Intraspinal injection of 3 c c normal horse serum.

Feb. 8, 12 noon: Intravenous injection of 50 c c of a 5% Berkefeld filtrate suspension of virus. At 12:30 p. m.: intraspinal injection of 3 c c of normal horse serum.

Feb. 9: Intraspinal injection of 3 c c normal horse serum.

Feb. 10: Intraspinal injection of 3 c c normal horse serum.

Feb. 11: 3 c c normal horse serum intraspinally.

Feb. 12: 3 c c normal horse serum intraspinally.

Feb. 13: Fine tremor of head.

Feb. 15: Hind legs weak.

Feb. 16: Coarse tremor. Flaccid paralysis of both arms. Prostrate in cage at 6 p. m. Paralyzed in all extremities.

Feb. 17, 9 a. m.: Moribund. Respiratory embarrassment. Monkey died at 8 p. m. Typical microscopic lesions of poliomyelitis.

Exper. 3.—Monkey 89. (Immune serum.) Feb. 7, 1918, 3:30 p. m.: Given 3 c c of fresh immune horse serum intraspinally.

Feb. 8, 11:30 a. m.: Intravenous injection of 50 c c of 5% virus suspension previously Berkefelded as above. Three c c immune horse serum intraspinally.

Feb. 9: 3 c c immune horse serum intraspinally.

Feb. 10: 3 c c immune horse serum intraspinally.

Feb. 12: 3 c c immune horse serum intraspinally.

Feb. 13: Intraspinal injection of 3 c c immune horse serum.

Feb. 14: Intraspinal injection of 3 c c immune horse serum.

March 22: Monkey remains well. Has never exhibited any symptoms of poliomyelitis.

Exper. 4.—Monkey 90. (Pooled immune monkey serum.) Feb. 7, 1918, 4 p. m.: Monkey received intraspinally 3 c c of fresh pooled immune monkey serum obtained by cardiopuncture from 2 monkeys convalescing from poliomyelitis.

Feb. 8: Intravenous injection of 50 c c of a Berkefeld filtrate suspension of virus. At 12 noon: Intraspinal injection of 3 c c immune monkey serum.

Feb. 9: Intraspinal injection of 3 c c immune monkey serum.

Feb. 10: Intraspinal injection of 3 c c immune monkey serum repeated.

Feb. 12, 13, and 14: Monkey received an intraspinal injection of 3 c c of immune monkey serum.

The clinical course of this animal is very instructive.

Feb. 20: Monkey appears to drag the left leg.

Feb. 22: Tremor and excitability. Definite weakness of both legs. Ataxia.

Feb. 23: Flaccid paralysis of all 4 extremities. Back muscles paralyzed. Monkey died at 1 p. m.

Sections of the pons, medulla, cervical and lumbar levels of the cord and of the intervertebral ganglia revealed marked lesions characteristic of poliomyelitis.

In these experiments both the immune horse and immune monkey serums were obtained Feb. 7 and used fresh without preservative. The pooled immune monkey serums were obtained from 2 monkeys, both of whom had survived a recent poliomyelitic infection with varying degrees of residual paralysis of 3-5 weeks' duration. The Control Monkey 88 receiving normal horse serum intraspinally developed a flaccid paralysis 8 days after intravenous injection of virus and died on the 9th day with marked characteristic lesions of poliomyelitis.

Monkey 90 receiving pooled immune monkey serum exhibited a delay of 9 days over Control 88 in the onset of the symptoms and died of typical poliomyelitis on the 10th day following the injection of virus.

Finally it is significent that only Monkey 90 treated by intraspinal injections of antipoliomyelitic horse serum remains well today and has never exhibited any symptoms of poliomyelitis.

SERIES 2

For these experiments a paralyzed monkey in the 1st day of complete prostration was etherized and a 5% suspension of the cord and medulla was prepared, shaken and centrifuged and the clear supernatent fluid injected intravenously according to the method of Amoss.

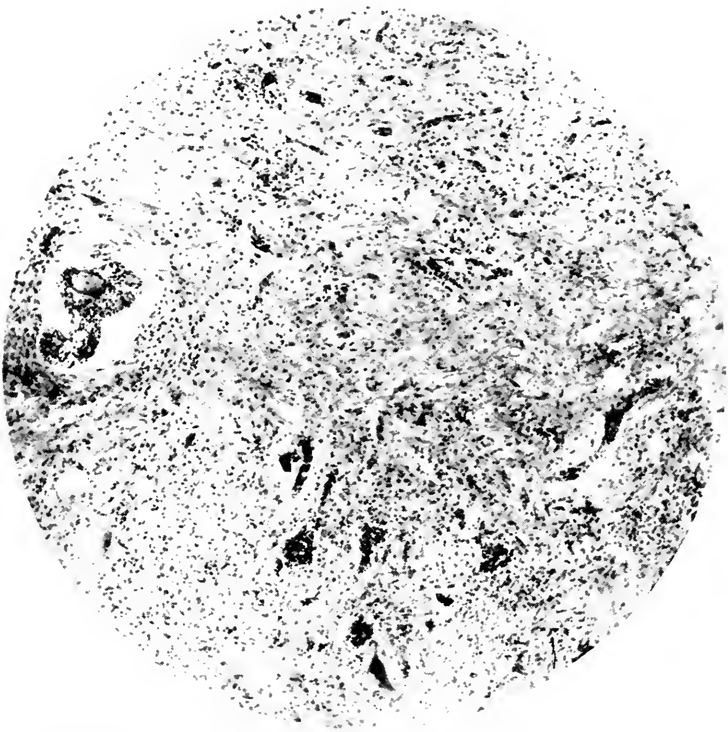


Fig. 1.—Monkey 93 (X70).—Typical neurophagocytosis, perivascular and interstitial lymphocytic infiltration in the anterior cervical horn of the spinal cord.

The immune horse serum was again obtained fresh the day of the experiments. The pooled immune monkey serum was obtained by cardiopuncture from 3 convalescent monkeys and divided into 2 equal lots. One lot was used fresh while the second was passed through a Berkefeld filter after previous preservation with 0.1% tricresol.

Exper. 5.—Monkey 91. (Control normal horse serum.) Feb. 18, 1918, 3:30 p. m.: Intraspinal injection of 3 c c of normal horse serum.

Feb. 19: Intravenous injection of 50 c c of a 5% suspension of virus. Followed by 3 c c normal horse serum intraspinally.

Feb. 20: Given 3 c c normal horse serum intraspinally.

Feb. 21: Intraspinal injection of 3 c c normal serum.

Feb. 22, 9 a. m.: Monkey has a complete flaccid paralysis of both arms and shoulders. Hind legs weak. P. m.: Prostrate in cage. Died at 8 p. m. same day.

Necropsy: Typical microscopic lesions of poliomyelitis in the brain and cord.

Exper. 6.—Monkey 92. (Pooled immune monkey serum.) Feb. 18, 1918, 5 p. m.: Intraspinal injection of 3 c c of fresh polled immune monkey serum.

Feb. 19: Intravenous injection of 50 c c virus suspension. Followed by 3 c c immune monkey serum intraspinally.

Feb. 20: 3 c c immune monkey serum as above.

Feb. 21: Given 3 c c immune monkey serum.

Feb. 22: 3 c c immune monkey serum intraspinally.

March 22: Monkey remains well.

Exper. 7.—Monkey 93. (Immune monkey serum filtered and preserved with 0.1% tricresol.) Feb. 18, 1918, p. m.: Given 3 c c of above immune monkey serum intraspinally.

Feb. 19: Intravenous injections of 50 c c of virus suspension. Given3 c c monkey serum intraspinally.

Feb. 20: Given 3 c c monkey serum intraspinally.

Feb. 21: Intraspinal injection repeated.

Feb. 22: Intraspinal injection repeated.

Feb. 23, a. m.: Generalized muscular weakness. P. m.: Paralyzed in all extremities and moribund. Died at 2 p. m.

Necropsy revealed marked lesions of poliomyelitis in the brain stem and lumbar cord.

Exper. 8.—Monkey 94. (Immune horse serum.) Feb. 18, 1918, 4 p. m.: Given an intravenous injection of 3 c c of fresh immune horse serum.

Feb. 19: Intravenous injection of 50 c c of fresh 5% virus suspension. Intraspinal injection of 3 c c immune horse serum.

Feb. 20: 3 c c immune horse serum intraspinally.

Feb. 21: 3 c c immune horse serum intraspinally.

Feb. 22: 3 c c immune horse serum intraspinally.

Feb. 28: Monkey remains well.

March 3: Left leg is weak. Monkey climbs awkwardly.

March 10: Monkey bright and active. Favors the left leg.

March 22: Monkey remains well. The muscles of the left leg are still weak. Prognosis as to ultimate complete recovery good.

An analysis of these experiments shows that the Control Monkey 91 treated with normal horse serum became completely paralyzed the 4th day and died the same day. Monkey 92 treated with fresh pooled immune monkey serum was protected against infection. Monkey 93 treated with the same lot of pooled immune monkey serum which was passed through a filter and preserved with 0.1% tricresol became severely paralyzed and died the 5th day following injection of virus. Monkey 94 treated with immune horse serum developed mild symptoms of infection 12 days after virus injection but was protected against a fatal termination and subsequently made a good recovery.

SERIES 3

All the monkeys (Ring Tails) in this series of experiments received an intravenous injection of 50 c c of a fresh 5% suspension of cord and medulla of a paralyzed monkey as in the experiments recorded in the foregoing.

Exper. 9.—Monkey 95. (Normal horse serum control.) Feb. 19, 1918: Given 2 c c normal horse serum intraspinally.

Feb. 20, 5 p. m.: Intravenous injection of 50 c c of a 5% suspension of virus. Intraspinal injection of 2 c c of normal horse serum.

Feb. 21: Intraspinal injection repeated.

Feb. 22: Given 2 c c normal serum intraspinally.

Feb. 23, 9 a. m.: Flaccid paralysis of both arms and shoulders. Died at 2 p. m.

Necropsy reveals characteristic microscopic lesions of poliomyelitis most marked in the brain stem and cervical cord.

Exper. 10.—Monkey 96. (Immune horse serum.) Feb. 19, 1918: Given intraspinal injection of 2 c c of fresh immune horse serum.

Feb. 20: Intravenous injection of 50 c c of fresh 5% virus suspension. Followed by intraspinal injection of 2 c c of immune horse serum.

Feb. 21: Given 2 c c immune serum intraspinally.

Feb. 22: 2 c c immune serum intraspinally.

Feb. 23: 2 c c immune serum intraspinally.

March 22: Monkey remains well.

Exper. 11.—Monkey 97. (Immune horse serum.) Feb. 19, 1918, 3:30 p. m.: Given an intraspinal injection of 2 c c of fresh immune horse serum.

Feb. 20: Intravenous injection of 50 c c of 5% virus suspension. Monkey received intraspinal injection of 2 c c immune horse serum.

Feb. 21, 22, and 23: Monkey was injected intraspinally with 2 c c of immune horse serum.

March 22: Animal remains well.

Under the conditions of the test the Control Monkey 95 treated by intraspinal injections of normal horse serum became severely paralyzed and died on the 4th day following the intravenous injection of virus. Monkeys 96 and 97 treated with intraspinal injections of immune horse serum remained well.

DISCUSSION

The striking results of the preceding experiments apparently demonstrate that fresh serum from the horse immunized for a considerable period with pure cultures of the coccus, the relation of which to poliomyelitis is under discussion, possesses definite power to prevent poliomyelitis in monkeys injected intravenously with massive doses of virus. Three monkeys were protected perfectly while the 4th monkey developed mild symptoms 14 days subsequent to injec-

tion with virus and recovered completely. The control animals injected with normal horse serum became severely paralyzed in all extremities and died as early as the 4th day after injection with virus.

A far more important point and one which would appear to invalidate the method as a practical test is brought out in the experiments outlined in Series 1. While the monkey receiving immune horse serum was protected perfectly against infection, Monkey 90 treated with fresh immune pooled serum from 2 recovered monkeys became severely paralyzed and died on the 10th day. It appears then that even specific immune monkey serum which is known to possess neutralizing properties for virus may not suffice to prevent infection under the conditions of this test. To overcome this apparent difficulty Amoss and Eberson employed "the mixed serums from several rhesus monkeys which had recovered from experimental poliomyelitis and subsequently received subcutaneous injections of the virus contained in the spinal cord and medulla (reinforced immune)."

Furthermore, the experiments in Series 2 show that while fresh pooled serum from 3 immune convalescent monkeys sufficed to protect Monkey 92 perfectly, this same serum after passage through a Berkefeld filter and the addition of 0.1% tricresol as a preservative acted in the manner of normal horse serum, facilitating a fatal poliomyelitic infection in Monkey 93 5 days subsequent to injection of virus. This apparent discrepancy may be explained by the observation of Flexner and Amoss that foreign serum and very probably tricresol when injected intraspinally act as irritants and set up an aseptic meningitis, thereby facilitating the passage of the virus from the blood into the central nervous system and promoting a poliomyelitic infection.

Accordingly, we have employed only fresh immune horse serum obtained the day of the experiments and used without preservative. It is reasonable to believe that guinea-pig complement and tricresol added to Rosenow's serum as tested by Amoss and Eberson may have produced an aseptic meningitis, thereby promoting a poliomyelitic infection. The accidental puncture of a vein while performing lumbar puncture often occurs and surely would permit the virus to pass directly into the spinal canal in considerable amounts. All of these factors when taken into consideration appear to condemn the method as a practical test of the therapeutic value of serum.

CONCLUSIONS

I have applied the therapeutic test, recently devised by Amoss and Eberson, to fresh samples of immune horse serum prepared by injections of the poliomyelitic coccus in the horse. Under the conditions of the test, 3 monkeys were completely protected while the 4th animal developed mild symptoms on the 14th day and subsequently recovered completely. The control monkeys receiving normal horse serum all developed a fatal poliomyelitis and died as early as the 4th day following injection of virus.

Fresh immune horse serum protected perfectly against infection while pooled immune monkey serum served only to delay the onset of a fatal infction.

The addition of 0.1% tricresol as a preservative to immune monkey serum sufficed to promote a fatal infection in one monkey. The same pooled immune monkey serum when used fresh without preservative protected perfectly against poliomyelitis.

The protective, neutralizing and curative properties of antipoliomyelitic horse serum as reported in previous publications serve to explain the beneficial results previously recorded in the serum treatment of poliomyelitis in man.

The apparent specific neutralizing properties of antipoliomyelitic horse serum for the virus as determined in vitro offers a convenient and satisfactory therapeutic test of the serum and seems to add additional proof of the etiologic relationship of the streptococcus to poliomyelitis.

CLINICAL AND EPIDEMIOLOGICAL STUDIES ON EPIDEMIC MENINGITIS

William H. Robey, Jr., M.D. (Boston), Major, M.R.C.
Howard L. Saylor, M.D. (Dayton), Captain, M.R.C.
Henry E. Meleney, M.D. (New York), Captain, M.R.C.
Hickman Ray, M.D. (Raleigh), First Lieutenant, M.R.C.
Gustav A. Landmann, M.D. (Scotland, S. D.), First Lieutenant, M.R.C.

From the Base Hospital, Camp McClellan, Ala.

CLINICAL

William H. Robey, Jr., and Howard L. Saylor

The group cases of epidemic meningitis which has occurred at Camp McClellan can in no sense be classed as an epidemic. The 26 cases which were admitted between Sept. 27, 1917, and April 4, 1918, are reported to contrast them with the meningitis cases of other cantonments. The history of the prodromal stage has been obtained in a sufficient number of cases to show great similarity in mode of onset. The majority of the cases began about 3 days before entrance with symptoms of coryza, general aching and stiffness, sometimes digestive disturbances often accompanied with projectile vomiting, and steadily increasing headache. There have been two epidemics resembling influenza in onset at this camp this year, one of which has been recently studied, in which similar prodromata have occurred and cases of meningitis at these times have been overlooked. It has been unusual to have a case of meningitis recognized before entrance. When there is not an epidemic and a regimental surgeon may not see more than 2 or 3 cases at great intervals, it is not surprising that even an increasing headache is overlooked. In every instance, as far as we have been able to discover, there was the history of gradual onset accentuating the belief that the disease is at first systemic and the cerebrospinal inflammation a later development. In not one case was the patient received at the hospital in whom the spinal fluid was not slightly turbid at the first puncture. One patient was in the hospital about 36 hours, being admitted during an epidemic characterized by symptoms suggesting influenza, before a diagnosis was made. The patient's symptoms were not unlike those of several others in the

Received for publication July 26, 1918.

ward except that the headache did not respond to the usual treatment, and there was very slight rigidity of the neck and a suggestive Kernig's sign. It has probably been the failure on the part of regimental surgeons to look carefully for neck rigidity and a beginning Kernig's sign which has delayed admission to the hospital. These two signs are the most reliable guides in distinguishing meningitis from other conditions with similar prodromata. In no case at the time of entrance have they been absent, although sometimes in so slight a degree that unless a careful physical examination was made the condition could be easily overlooked. A rash occurred in only one instance.

In any patient with the foregoing history and even a slight rigidity of the neck and suggestive Kernig's sign, lumbar puncture has been done at once. The fluid was withdrawn under antiseptic precautions and its character and degree of pressure carefully noted. Whether the fluid was cloudy or not, 40 c c of antimeningococcic serum was injected and the fluid in the meantime was sent to the laboratory for study. The injection was repeated in 8 hours and again in from 8-12 hours according to the urgency of the symptoms. Several injections at frequent intervals have generally relieved all symptoms and subsequent treatments have been given at 24-48 hour intervals according to the reappearance of cerebrospinal symptoms. Intravenous treatment has not been used because all our cases had become cerebrospinal in type by the time of admission. Rockefeller Institute polyvalent serum has been used in practically all of the treatments and the response has been immediate in most of the cases. There were a few patients in whom spinal puncture was done with negative results, but one injection of serum was made at the time as a precautionary measure. They are not included in the series since bacteriologic examination was negative and the clinical course was not that of meningitis.

Relapses occurred in Cases 4, 20, and 26. In Case 4, the patient was normal for 1 week and then relapsed. In Case 20, the patient had been normal for 22 days, was free of meningococci, and had been transferred to another ward when all symptoms returned and the spinal fluid became cloudy. Treatment was begun again with excellent recovery. One injection of 40 c c cleared the fluid of meningococci. In Case 26, the patient had a relapse and died after several days. In cases having a poor drainage or a relapse with symptoms of

TABLE 1

SUMMARY OF CLINICAL FEATURES

Case Number	Mode of Onset	Duration at Entrance	Rash	Objective Symptoms	Type of Organism	Spinal Fluid	Treatment	End Results	Remarks
1	10/ 9/17 C.; V.	1 day	None	Loss of consciousness	Meningococcus not determined	Turbidity present	F.R. 92 S. 75	Recovered 11/21/17	Slight deafness
2	10/16/17 Unconscious on admission		None	Delirium; neck rigid	0	Turbid	F.R. 150 S. 150	Recovered 12/7/17	Serum sick
3	10/21/17 H.; V.	2 days	None	Tinnitus aurium; opisthotonus; Kernig; stiff muscles	Meningococcus not determined	Turbid	F.R. .. S. 135	Recovered 1/30/18	Total deafness
4	11/17/17 C.; H. sore throat	3 days	None	Kernig; stiff neck	Meningococcus not determined	Turbid	F.R. 190 S. 140	Recovered 1/28/18	Preceded by talipes equinus and bronchitis
5	11/29/17 V.; H.; abdominal pain	2 days	None	Opisthotonus; Kernig; muscular twitching	Meningococcus not determined	Turbid	F.R. 110 S. 80	Recovered 2/12/18	No sequelae
6	12/22/17 Unconscious		None	Opisthotonus; Kernig; delirium; incontinence of urine; pupils dilated	Meningococcus para	Turbidity pressure+	F.R. ... S. 155	Died 12/24/17	Severe toxemia
7	12/30/17 H.; pain in right knee	2 days	None	Kernig; rigid neck	Meningococcus normal	Turbid	F.R. 190 S. 175	Recovered 2/12/18	Deaf in right ear, improving
8	12/31/17 H.; constipated	3 days	None	Kernig; stiff neck	Meningococcus para	Turbidity pressure+	F.R. ... S. 200	Recovered 2/6/18	No sequelae
9	1/ 1/18 V.; H.; pain in abdomen	3 days	None	Opisthotonus; Kernig; unconscious	Meningococcus normal	Turbidity pressure+	F.R. 195 S. 120	Recovered	No sequelae; preceded by tonsillitis, scarlet fever
10	1/1/18		None	Opisthotonus; Kernig; stupor	Meningococcus para	Turbidity pressure+	F.R. 240 S. 170	Recovered 2/9/18	Diplopia improving
11	1/3/18 V.; H.	2 days	None	Opisthotonus; Kernig	0	Turbid	F.R. 420 S. 320	Recovered 2/7/18	No sequelae
12	1/6/18 V.; H.	3 days	None	Kernig; stiff neck	Meningococcus normal	Turbidity pressure+	F.R. 275 S. 210	Recovered 2/12/18	Diplopia improving
13	1/7/18 H.; V.; stupor	2 days	None	Kernig; neck stiff	Meningococcus normal	Turbid	F.R. 150 S. 120	Recovered 2/15/18	No sequelae
14	1/22/18 H.; V.; stupor	2 days	None	Opisthotonus; Kernig; delirium	Meningococcus not determined	Turbidity pressure+	F.R. 200 S. 340	Recovered 2/12/18	No sequelae; civilian
15	1/22/18 H.: bronchitis	2 days	None	Kernig; unconscious	Meningococcus autopsy	....	....	Died 9 hours	Severe toxemia
16	1/24/18		None	Unconscious; Kernig; rigid neck; bronchitis	Meningococcus normal	Turbidity pressure+	F.R. 130 S. 130	Died 1/27/18	Severe toxemia
17	1/24/18 neck stiff; Kernig	2 days	None	Opisthotonus; severe headache	Meningococcus not determined	Turbidity pressure+	F.R. 300 S. 320	Recovered	No sequelae
18	1/26/18 C.; H.; V.	3 days	None	Delirium; Kernig	Meningococcus normal	Turbidity pressure+	F.R. 685 S. 560	Recovered 3/10/18	No sequelae

Case numbers in clinical record do not agree with numbers in laboratory table.
C = chill, V = vomiting, H = headache, F.R. = fluid removed, S = serum in c c.

TABLE 1—*Continued*
SUMMARY OF CLINICAL FEATURES

Case Number	Mode of Onset	Duration at Entrance	Rash	Objective Symptoms	Type of Organism	Spinal Fluid	Treatment	End Results	Remarks
19	1/28/18 C.; H.; V.; tremor	2 days	None	Unconscious; convulsions; opisthotonus; Kernig	Meningo-coccus normal	Turbidity pressure+	F.R. 605 S. 530	Recovered	No sequelae
20	2/11/18 H.; V.; C.	3 days	Hemorrhagic over whole body	Stiff neck; stupor; Kernig	Meningo-coccus normal	Turbidity pressure+	F.R. 160 S. 200	Recovered 3/12/18	No sequelae
21	2/21/18 H.; V.	2 days	None	Pupils undilated; semiconscious; Kernig	Meningo-coccus not determined	Turbidity pressure+	F.R. 370 S. 240	Recovered	Spinal fluid + after 22 days
22	3/19/18 Stiff neck; H.	2 days	None	Very slight Kernig	Meningo-coccus not determined	Turbidity pressure+	F.R. 110 S. 110	Recovered	No sequelae
23	4/9/18 C.; H.; V.	2 days	None	Unconscious; Kernig	Meningo-coccus normal	Turbidity pressure+	F.R. 280 S. 280	Recovered	No sequelae
24	3/24/18 C.; H.; V.; deafness	2 days	None	Unconscious; opisthotonus; deafness; Kernig	Meningo-coccus para	Turbidity pressure+	F.R. 970 S. 640	Recovered in H.	Pericarditis of 8 days' duration
25	4/16/18 C.; H.	4 days	None	Neck and back stiff	Not determined	Turbidity pressure+	F.R. 870 S. 620	Recovered	No sequelae
26	4/10/18	5 days	None	Opisthotonus; unconscious; Kernig	Meningo-coccus para	Turbidity pressure+	F.R. 320 S. 240	Died 5/2/18	Relapse 8 days, heart murmur disappeared after 5 days

sepsis the question of ventricular puncture arises. Cases 4, 9, 20, and 26 were patients in whom this form of treatment might have been tried. All patients, excepting Case 26, recovered and without sequelae.

DESCRIPTIONS OF CASES SHOWING UNUSUAL FEATURES

CASE 2.—Patient admitted in wild delirium requiring several men to hold him in bed. There was only slight rigidity of neck and no Kernig signs. About 3 hours after entrance 30 c c of cloudy fluid were withdrawn and an equal amount of serum was given. Two days later the patient was semirational but lay in marked opisthotonos. Five days from entrance perfectly rational, rigidity of neck less but still quite marked. Ten days, the general condition good: slight deafness, right deltoid and pectoralis muscles completely paralyzed and the brachialis anticus weak. Eleven days, marked urticaria and other signs of serum sickness of a few days duration. Recovery from this time was uninterrupted save for the paralyses described which persisted at the time of his discharge from the army, Dec. 7, 1917.

CASE 3.—Admitted Oct. 21, 1917. Taken ill 24 hours before with headache and general aching. He complained of tinnitus aurium at entrance. Conscious but slightly confused. Neck rigid but Kernig sign present. Two days later partially deaf, marked Kernig sign and absolutely deaf. Subsequent course was uneventful except that the deafness persisted: ear drums normal. Total deafness due to involvement of 8th nerve. Discharged from the army, Jan. 30, 1918.

CASE 4.—Admitted Nov. 17, 1917, at 11 p. m. Complained of sore throat and severe headache. For one week before entrance had a coryza and the night before had a chill and could not get warm. There was slight stiffness of the neck and suggestive Kernig sign of both legs; 40 c c of cloudy fluid were removed and 20 c c of serum given. The next day the patient was more comfortable. Meningococci found. The patient improved until Nov. 24 when there was a return of headache and a left orchitis developed. Jan. 6, left parotid enlarged and two days later became bilateral. During this time patient lay in a stupor, lost greatly in weight, and looked as though he was going to die. There was diplopia and deafness but special examinations showed the eyes and ears to be normal. There was incontinence of urine and feces. As late as Dec. 19 the spinal fluid was cloudy. Treatment during the latter part consisted in withdrawal of spinal fluid from time to time. On Dec. 25 there was a sudden cessation of symptoms and recovery was uninterrupted from that time. Discharged to duty Jan. 28, 1918, in excellent condition.

CASE 9.—Admitted Nov. 16, 1917, with tonsillitis. Dec. 1, a moderate course of scarlet fever began. Jan. 1, 1918, the patient was to have been discharged to duty from scarlet fever ward, having been up and in good condition for 2 weeks. The night before he had pain in the abdomen, some nausea, and vomited at 8 a. m. At 9 a. m. he became suddenly unconscious. Supraorbital pressure and pin pricks failed to arouse him. The pupils were equal and reacted normally. It was thought that there might be an embolus. There was slight rigidity of the neck and double Kernig sign was very slight. In 2 days the patient was practically normal again except for a degree of fever when marked rigidity of the neck returned with marked double Kernig sign. Spinal puncture revealed a slightly cloudy fluid under considerable pressure. Meningococci present. For 2 weeks the patient's condition was unfavorable; low muttering delirium with incontinence occurred. Serum given amounted to 120 c c at intervals according to symptoms. On Jan. 9 the temperature became normal and progress was steady from that time. Excellent recovery and returned to duty. There had been no other case of epidemic meningitis in the same ward. Readmitted May 22 with spastic paraplegia manifested by slight ataxia, exaggerated knee jerks, marked ankle clonus and double Babinski. No sphincter involvement.

CASE 20.—This patient was admitted as one of "fever undetermined," yet by the time he was entered at the hospital the ward surgeon had no trouble in making the diagnosis. He was unconscious, with rigidity of the neck and double Kernig sign. Although unconscious at entrance, this man, after 40 c c serum, was sufficiently conscious at the end of 18 hours to tell his name and organization and made an uninterrupted recovery. He was the only patient in the series who showed a rash.

CASE 26.—Five days before entrance on April 10, 1918, this patient felt stiff. Muscles and arms and legs severely stiff; moderate headache; nausea and vomiting of the projectile type. Unconscious at entrance, rigidity of the neck and double Kernig sign. This patient never had a normal temperature except from the second to the fifth day after entrance although there was always more or less opisthotonos. On the 15th, the spinal fluid became clear and except for the opisthotonos the general condition was much improved. On the 22nd, the patient became unconscious again. The spinal fluid ran well at first, then became cloudy, then purulent. Incontinence of urine and feces. The fluid became thicker and harder to withdraw and the back pressure was considerable.

making it difficult to introduce the serum. April 28 and 29 the patient showed marked improvement, regained consciousness, understood and answered questions and took and retained food. On May 1 he became profoundly unconscious and died on May 2.

SUMMARY

Total number of cases, 26; recoveries, 22, or 85%; deaths 4, or 15%.

Total number of cases which received serum treatment, 25, with 22, or 88%, recoveries.

Twelve patients were unconscious on admission or very soon thereafter, of whom 3 died.

Meningococcus, normal type, found in 9 cases; para type in 5 cases, and not determined in 12 cases.

Smallest amount of fluid removed, 90 c c; largest amount, 970 c c; average amount, 325 c c.

Smallest amount of serum injected, 75 c c; largest amount, 640 c c; average amount, 265 c c.

Three cases received more serum than amount of fluid removed: one, F.R., 200 c c—S., 340 c c; one, F.R., 300 c c—S., 320 c c; one, F.R., 480 c c—S., 560 c c.

Sequelae.—Deafness: Four patients, of whom one was total.

Diplopia: Two, both patients recovering rapidly when sent to duty.

Pericarditis: One, who is now recovering in the hospital, but still has rapid pulse.

Paraplegia: One (Case 9), who developed the trouble about a month after being sent to duty.

It will be noted that the prevailing symptoms of onset of disease were a chill or a cold feeling, severe headache which was many times associated with pain in the back and joints, and vomiting of the projectile type. These symptoms always preceded the rigidity of the neck muscles and Kernig's sign and add greatly to the theory that the disease is systemic before the advent of the cerebral and spinal symptoms.

In Case 16, several punctures did not obtain fluid and as these were made high up the necropsy bears out the fact that the seropurulent character of the fluid was responsible therefor. Cases 6 and 15 were no doubt overcome by the severity of the toxemia existent.

As many as 6 cases came from one detachment and the balance of the cases were quite widely scattered over the Division. Meningitis serum was depended on almost solely for results and no sedatives

were given except occasionally to control pain after a puncture and administration of serum. Later some bromids and potassium iodid were given in small doses. Codein was used in preference to morphia and given hypodermatically. Temperatures ranged around 102 F. and higher than 103 F. was exceptional. In all cases, with one exception (Case 23), the pupils were normal in size and reacted very freely to light until the disease was unmistakably established. Several patients complained of photophobia during convalescence and most of them retained some rigidity of the muscles late into convalescence.

EPIDEMIOLOGICAL

HENRY E. MELENEY, HICKMAN RAY,
AND
GUSTAV A. LANDMANN

The occurrence of meningococcus meningitis among the troops at the various army cantonments during the past winter has led to the culturing of a vast number of men for the purpose of detecting the carriers responsible for the spread of the disease. This work has been done in a variety of ways at different cantonments, both as regards technic and the size and character of the groups of men cultured. The types of the disease met with and the extent of the epidemics have also been quite varied. How much all the culturing has accomplished in preventing and checking epidemics is impossible to determine, for the factors entering into the development of the disease in those who acquire the organism in their respiratory passages are as yet but poorly understood. Neither has the organism been finally classified into its various subgroups, nor the pathogenic types separated from the nonpathogenic, if the latter exist. It is, therefore, desirable to record the results of work with this organism in order to help simplify the problems connected with it. At Camp McClellan there have been so far comparatively few cases of meningitis, but a great deal of culture work has been done to detect carriers. It is the purpose now to present (1) some of the results obtained in identifying the various types of meningococcus, and (2) some points of interest in the epidemiology of the subject.

To March 31, 1918, 23 cases of meningococcus meningitis occurred at Camp McClellan; 22 of these received serum treatment; the other

patient died before serum could be administered. Of the 22 treated cases, 19 have already recovered and 1 is convalescent. Two cases were fatal despite several intraspinal treatments with serum. The mortality has therefore been 9.1% in treated cases. In the same period of time 10,076 nasopharyngeal cultures were taken to detect meningococcus carriers, and 934 carriers were found, or 9.3% of the total number of cultures taken. Some men were cultured more than once which would raise the percentage of carriers to total men examined slightly above 10%. Nasopharyngeal cultures were taken on whole companies in which cases of meningitis occurred, and also, after Dec. 30, 1917, on all men who had been away on furlough or who entered the camp as new troops.

Methods

Culture Medium.—Veal infusion agar, 0.3-0.5% acid to phenolphthalein, with 0.5% dextrose and 5% horse serum added, was used for both plates and slants. This medium was so favorable for the meningococcus that on the plates a 24-hour growth would produce a colony 0.5-1.5 mm. in diameter. When the officer examining the plates became accustomed to their appearance on this medium, confusion with the influenza bacillus, the streptococcus and other organisms was an uncommon occurrence. The use of whole or laked blood for the plates, as is generally advocated[1] instead of serum, was therefore deemed inadvisable, as serum was easier than blood to keep on hand for constant use.

Cultures.—When a case of meningococcus meningitis developed in a certain company, the company was at once placed in quarantine, either in its own company street or in a detention camp. The whole company was then cultured as rapidly as possible, usually all on the same day. After isolating the carriers discovered by the first cultures, one of two procedures was followed. In the early months of our work all the men whose plates contained suspicious gram-negative diplococci which did not agglutinate, were recultured. In the later months this procedure was abandoned, but all men who were tentmates of men discovered to be carriers were recultured, and this process was repeated until no more carriers were found. Despite the high percentage of carriers isolated, we believe that neither of these methods discovered all the carriers in a given company, for some must have escaped detection on the first culture. In the case of men returning from furlough, one culture only was taken.

All cultures were taken by a medical officer from the Base Hospital Laboratory. The West tube swab was used entirely. At first a separate petri plate was used for each culture, but later 2 cultures were taken on a small plate (10 cm. diameter), and 4 on a large plate (15 cm. diameter). It was found that this method, when carefully used, gave results as accurate as with 1 culture to a plate. It is only to be recommended, however, when a very large amount of work has to be done with a minimum of effort, for there is always danger of carrying a few organisms from one side of the plate to the other. After streaking the plate in one small area and around the rim with the inoculated swab, the culture was spread with another sterile cotton swab, which gave a very

[1] Flexner, S.: Jour. Am. Med. Assn., 1917, 69, p. 639.

satisfactory distribution of the colonies for fishing, and a much better distribution than could be secured with a platinum loop. The plates were transported in a large sheet-iron water jacket with compartments for 100 plates. After 24 hours' incubation, all the plates were inspected by one officer. He discarded and reported as negative the plates which contained no suspicious colonies. The remaining plates were then distributed among the officers doing the meningitis work, and the suspicious colonies were transplanted to serum-dextrose-agar slants, usually one colony from each plate. The slants were then incubated for 24 hours, an emulsion in salt solution made of the growth, and a drop of the emulsion stained and examined to prove the purity of the culture.

Agglutination Tests.—For the routine agglutination tests Rockefeller polyvalent antimeningococcus horse serum was used in 1:200 dilution. Mulford's normal horse serum in 1:50 dilution, and normal salt solution were used as controls. The agglutination tests were placed in pans of water in a 55 C. inactivator over night and were read the next morning. For a short period in January a 1:100 dilution of polyvalent serum was used for agglutination tests, but parallel tests with a 1:200 dilution showed that all cultures giving clearcut (2 plus) reactions with the 1:100 dilution, also gave positive reactions (1 plus or 2 plus) with the 1:200 dilution. At that time about 15% of the cultures were giving positive agglutination tests with the 1:200 dilution. This percentage was so high that it was considered probable that the test was identifying all true meningococci, and that the 1-100 dilution was probably agglutinating some organisms which were not true meningococci. The higher dilution was therefore readopted for routine work.

Parallel with the polyvalent agglutinations, monovalent agglutinations with rabbit serum furnished by the Army Medical School against Rockefeller Strains 1 (Normal), 10 (Intermediate A), 30 (Intermediate B), and 60 (Para), were made on 173 cultures. The titer of the 4 serums against their respective organisms was stated by the Army Medical School to be as follows:

Normal (Strain 1)	1:1600
Intermediate A (Strain 10)	1:800
Intermediate B (Strain 30)	1:800
Para (Strain 60)	1:1600

Some of these tests were done with both polyvalent and monovalent serum in 1:100 dilution, some with polyvalent in 1:200 and monovalent in 1:100 dilution, some with both in 1:200 dilution. These different combinations were used in order to determine which would detect the largest number of true meningococci, and at the same time differentiate their types most distinctly.

RESULTS OF MONOVALENT AGGLUTINATIONS

Most of the 173 cultures on which monovalent agglutinations were done, agglutinated with both the polyvalent serum and one of the monovalent serums. There were some, however, which agglutinated with the polyvalent serum, but with none of the monovalent serums, and others which agglutinated with one monovalent serum, but not with the polyvalent serum. Table 1 shows these results.

The first part of this table shows 100 cultures which agglutinated with both polyvalent and monovalent serum; 53 of these were of the

Normal (Strain 1) type, 3 were Intermediate A (Strain 10), 5 were Intermediate B (Strain 30), and 39 were Para (Strain 30). Thus the Normal type is seen to have predominated among the carriers, with the Para type second, and the two Intermediate types quite uncommon. The same proportions existed among the types of organisms recovered from the spinal fluid of our cases, as will be shown later.

TABLE 1

SUMMARY OF POLYVALENT AND MONOVALENT AGGLUTINATIONS

—	Polyvalent + Monovalent +	Polyvalent + Monovalent 0	Monovalent + Polyvalent 0	Totals
Polyvalent 1:100 Monovalent 1:100	22	19	1	42
Polyvalent 1:200 Monovalent 1:100	34	0	7	41
Polyvalent 1:200 Monovalent 1:200	44	34	12	90
Totals	100	53	20	173

The second part of Table 1 shows 53 cultures which agglutinated with the polyvalent serum but with none of the monovalent serums. It will be noticed that all of these occurred when both polyvalent and monovalent serum were used in the same dilution, 19 of them when both were used at 1 : 100, 34 when both were used at 1 : 200. In no case did it occur when the polyvalent serum was used at 1 : 200 and the monovalent at 1 :100. These facts suggest that there are some meningococci which were detected more readily by our polyvalent than by our monovalent serum, when both were used in the same dilution, but whose type could be determined if the monovalent serum were used in high enough concentration. It is, of course, impossible to be sure which type these organisms would have fallen into, had we been able to agglutinate them with a monovalent serum. When the polyvalent serum was used at 1 :100, some of the organisms which agglutinated may not have been true meningococci. There is evidence, however, that this was not always the case, and that it was the para type of meningococcus which sometimes failed to be agglutinated by the Type 60 (para) monovalent serum. The following points suggest this: Of the 19 cultures which agglutinated only with the polyvalent

serum when all serums were used in 1:100 dilution, 16 were in a series of 35 agglutination tests done on cultures from men in companies where a para type of organism had caused meningitis. Sixteen other cultures of this 35, proved to be para organisms. The remaining 3 were of the normal type. Thus there was a great preponderance of the para type in these companies. Furthermore, 1 culture from which 2 colonies were transplanted, produced from 1 colony agglutination with both polyvalent and para monovalent serum, and from the other colony, agglutination with polyvalent serum alone. Again, 3 men whose cultures in this series contained a para organism, on later culture, when all serums were used in 1:200 dilution, showed an organism agglutinating only with the polyvalent serum. We have noticed also that when agglutination does occur with the para monovalent serum, it is usually much less complete than the agglutination with the polyvalent serum. Although most of this evidence is circumstantial, it points to the fact that the monovalent rabbit serum with which we worked, was less able, in the dilutions in which it was used, to agglutinate all meningococci of the para type than was the polyvalent horse serum. The explanation for this undoubtedly is that the polyvalent serum is from an animal immunized against several organisms of the para type, whereas the monovalent serum is from an animal immunized against the Strain 60 organism alone. Possibly a 1:50 dilution of the para monovalent serum would have agglutinated the organisms which failed to agglutinate with the 1:100 dilution. We tried a 1:50 dilution of the 4 monovalent serums for typing, but found that when this was done the agglutination of many organisms was complete with more than 1 serum, which made it difficult to decide to which type the organism belonged. With a 1:100 dilution, however, a stronger agglutination was always secured with 1 serum than with any of the others, which made that dilution much better for routine work. Our results suggest that possibly a fifth monovalent serum is needed to identify all meningococci in routine army work, or that the Type 60 serum should be replaced by one with better agglutinating power for all the members of the para group.

Now, turning to the third part of Table 1, it will be seen that 20 of the 173 cultures on which monovalent agglutinations were done agglutinated with 1 monovalent but not with the polyvalent serum. Their distribution among the 4 types is shown in Table 2.

TABLE 2
CULTURES AGGLUTINATING WITH MONOVALENT SERUM, ONLY

	Normal (1)	Intermediate A (10)	Intermediate B (30)	Para (60)	Totals
Polyvalent 1:100 Monovalent 1:100	1	+	+	+	1
Polyvalent 1:200 Monovalent 1:100	5	2	+	+	7
Polyvalent 1:200 Monovalent 1:200	10	1	+	1*	12
Totals..	16	3	0	1	20

* Agglutination of this culture was incomplete (one plus).

It will be seen from this table that 80% of the organisms that agglutinated with a monovalent but not with the polyvalent serum were of the normal (Strain 1) type. Unfortunately no series of monovalent agglutinations was done on cultures from a company where a normal type of meningococcus had caused meningitis. It is therefore not known how many carriers harboring this type of meningococcus were missed because of not using the monovalent serums in routine agglutinations. Only 1 culture failed to agglutinate with polyvalent serum when it was used in 1 : 100 dilution, showing that that dilution identified nearly all true meningococci, but we suspect that this dilution also agglutinated some organisms which were not true meningococci. The most interesting point brought out by this table, however, is that it was almost invariably the normal type of meningococcus which was found when the monovalent rabbit serum produced agglutination and the polyvalent horse serum did not. We cannot assert that this shows any weakness of the polyvalent serum against meningococci of the normal type, although we at first failed to get polyvalent agglutination of 1 spinal fluid organism from a fatal case (Case 6), even in a 1 : 100 dilution of the serum. All we can say is, that whatever it is that interferes with agglutination by the polyvalent serum, interferes with organisms of the normal type, not with those of the para type.

Another point of interest in connection with these monovalent agglutinations is the cross-agglutination which frequently occurs. Used in either 1 : 100 or 1 : 200 dilution, most organisms which are agglutinated by the normal (Strain 1) serum are also, to a lesser

degree, agglutinated by the intermediate B (Strain 30) serum. And most organisms which are agglutinated by the para (Strain 60) serum are also, to a lesser degree, agglutinated by the intermediate A (Strain 10) serum. In our work we have classified the various organisms according to the type of serum with which the most complete agglutination was produced. The difference has always been distinct enough in the 1:100 dilution of the serum to leave no doubt in our minds to which type a given organism belonged. This power of cross-agglutination suggests that the so-called normal type and the intermediate B type are closely related, and that the intermediate A type and the para type are likewise closely related. If this is true the present nomenclature does not accurately express the relationship of the 4 strains of meningococci, and the 2 intermediates should be reversed.

VARYING AGGLUTINABILITY OF ORGANISMS

We frequently found that different cultures from the same carrier, or even different colonies from the same plate, varied in their agglutinability with polyvalent and monovalent serum. This occurred in spite of the fact that the technic of running through the tests was as uniform as possible, and the gross and microscopic appearances of the cultures were similar. Examples of this among the para group of organisms were cited in the foregoing. Similar examples were observed among the normal type. It is interesting to note, however, that where the agglutinability varied in an organism of the para type, it was always the para monovalent serum which failed to agglutinate, when the polyvalent serum did agglutinate. On the other hand, when the agglutinability varied with an organism of the normal type, it was always the polyvalent serum which failed to agglutinate, when the normal monovalent serum did agglutinate.

An example of this variability was met with in the spinal fluid organism from the fatal case of meningitis mentioned above (Case 6). This organism at first failed to agglutinate with the Rockefeller polyvalent serum even in a 1-100 dilution, although agglutination was produced by the normal monovalent serum in 1-400 dilution. The organism was sent to Dr. Olitsky at the Rockefeller Institute, who succeeded in agglutinating it from a subculture on plain dextrose agar, by the Rockefeller polyvalent serum in 1:200 dilution, as well as by the normal monovalent serum in 1:400 dilution. We also succeeded, after several generations of subculture, in agglutinating it from serum-

dextrose-agar with Rockefeller polyvalent serum in 1:200 dilution. Repeated transplantation apparently increased the agglutinability of the organism. Whether the presence of the horse serum in the medium with which we worked had anything to do with this varying agglutinability we do not know. With our volume of work it was impracticable to use any other kind of serum for our medium or to do our agglutinations on cultures grown on plain dextrose agar.

SUMMARY OF MONOVALENT AGGLUTINATIONS

Summing up our observations on monovalent agglutination tests, we would emphasize the following points:

1. A potent polyvalent antimeningococcus horse serum in 1:100 dilution will identify nearly all true meningococci, but may agglutinate some organisms which are not true meningococci.

2. Four monovalent antimeningococcus rabbit serums, against Normal, Intermediate A, Intermediate B, and Para strains, respectively, if used in strong enough concentration, will probably identify all meningococci, but a 1:100 dilution is the strongest which will easily differentiate between the types, and this dilution fails to agglutinate some true meningococci.

3. Some normal type meningococci agglutinate more readily with the monovalent than with the polyvalent serum, and some para type meningococci agglutinate more readily with the polyvalent than with the monovalent serum.

4. It is therefore suggested that to obtain the most accurate results in identifying and typing meningococci from the nasopharynx, a polyvalent horse serum in 1:200 dilution and 4 types of monovalent rabbit serum in 1:100 dilution should be used.

THE RELATION OF CASES TO CARRIERS

The relation of the meningococci from our cases of meningitis to the meningococci from the carriers in the companies in which the disease occurred, is shown in Table 3. Only the cases in which the organism causing the disease was typed are included in this table. In the 11 other cases of meningitis, either no organism was recovered on culture of the spinal fluid, or, if recovered, the organism was not typed.

This table, although very incomplete, is quite definite as far it goes. It will be seen that all the cases of meningitis occurring in the 112th

Heavy Field Artillery, whose organisms were typed, were caused by a meningococcus of the para type, while all the other cases except one, whose organisms were typed, were caused by the normal type. The companies from which Cases 2 and 3 came are the only ones on whom a regular series of monovalent agglutinations was done. The table shows very definitely the predominance of the para type of meningococcus in these companies. It is unfortunate that no series of monovalent agglutinations were done on cultures from companies in which the normal type had caused meningitis. It is probable that in these companies the normal type of meningococcus predominated.

TABLE 3
MENINGOCOCCI FROM CASES AND ASSOCIATED CARRIERS

Lab. Case No.	Co.	Regt.	Spinal Fluid Organism	Organism from Carriers					
				Normal (1)	Inter. A (10)	Inter. B (30)	Para (60)	Poly + Mono 0	Mono + Poly 0
1*	A	112 HFA	Para						
2	E	112 HFA	Para				14	1	
3	Sup	112 HFA	Para	3			8	1	1
5*	A	104 MP	Para						
6	C	111 FA	Normal	1		1			
7	4	104 AT	Normal		1			1	
10*	D	116 Inf.	Normal						
12	I	116 Inf.	Normal	1					
14	D	104 Eng.	Normal	2			1	1	
15*	E	104 Eng.	Normal						
19*	..	113 Amb.	Normal						

* No monovalent agglutinations done.

An attempt has been made to connect each case of meningitis with the presence of a chronic meningococcus carrier who was in direct contact with him. This has been possible to a considerable extent, as is shown in Table 4. Only the most chronic carrier in each company, or more transient carriers who were in close contact with the cases, have been entered in this table.

It will be noted that 14 of the 23 cases came into direct or indirect contact with a carrier who continued to harbor the meningococcus for over a month after detection, despite the spraying of his nasopharynx with an antiseptic 4 times a day. Six of the cases came into frequent direct contact with these chronic carriers, and Cases 16 and 17, which started within a week of each other, were in direct contact with the same chronic carrier. This evidence seems to point definitely to the chronic meningococcus carrier as the source of this series of cases.

TABLE 4
RELATION OF CASES TO CHRONIC CARRIERS

Lab. Case No.	Co.	Regt.	Spinal Fluid Organism	Carrier in Contact: Carrier No.	Carrier in Contact: Origin	Carrier in Contact: Degree of Contact	Duration of Carrier State
1	A	112 FA	Para	207	?	None known	43 days
2	E	112 FA	Para	473	Para	None known	34 days*
				156	?	Tent mate	Less than 17 days
3	Sup.	112 FA	Para	209	?	Tent mate 2 weeks	53 days
4	Hdq.	112 FA	?	113	Para	Tent mate 1 week	30 days†
5	A	104 MP	Para	No carriers detected			
6	C	111 FA	Normal	490	Normal	Next tent, friend	17 days
7	4	104 AT		189	?	None known	63 days
				243	?	Tent mate	35 days
				227	?	Tent mate	Less than 21 days
8	A	116 Inf.	?	184	?	Visited tent	51 days
9	C	116 Inf.		0	?	Case=Clerk of Co. Carrier=Capt.	19 days
				153	?	None known	29 days
10	D	116 Inf.	Normal	—	—	—	—
11	F	116 Inf.	?	66	?		40 days
12	I	116 Inf.	Normal	120	Normal	None known	89 days†
13	M	116 Inf.	?	104	?	None known	65 days
				322	Normal	None known	28 days*
14	D	104 Eng.	Normal	657	Normal	None known	42 days
				193	?	Tent mate	7 days
				195	?	Tent mate	7 days
15	E	104 Eng.	Normal	Company not cultured			
16	309	Bakery	?	190	?	Cpl. of squad	64 days
17	309	Bakery	?	190	?	Cpl. of squad	64 days
18	B	113 Inf.	?	130	Normal	None known	45 days*
19	113	Amb. Co.	Normal	603	?	Next tent	26 days
				604	?	Company barber	17 days
20	H	114 Inf.	?	758	Normal	None known	38 days†
21	I	114 Inf.	?	Company formed only 5 days before onset			
22	..	3rd. FA	Normal	Case imported‡			
23	—	—	?	Case a civilian carpenter			

* Meningococcus again obtained from carrier, April 3, 1918.
† Carrier not yet released from quarantine.
‡ Case found to be a meningococcus carrier on return from furlough following recovery. Had had 3 negative nasopharyngeal cultures before discharge from hospital. Carrier organism also of normal type.

There is not space in this paper for a detailed discussion of the chronic meningococcus carrier in his relation to cases of meningitis. The subject is one which needs considerable study. The carriers at Camp McClellan were isolated in a quarantine camp and were sprayed by way of the nose and mouth 4 times a day. Both dichloramin-T and carbolized solution of iodin, N. F. (Boulton's solution) were used for the spray. One worked about as well as the other. Some carriers cleared up quickly; others seemed to be unaffected by the treatment. Sunshine and warm fresh air apparently did more than spraying to rid the nasopharynx of the meningococcus. Cultures were taken at 3-day intervals and the carrier released after the third consecutive negative culture. The men were not sprayed within 12 hours before a culture was taken. We were not convinced that this procedure

would permanently rid the chronic carriers of the organism, so about a month after their release from quarantine 30 of the most chronic carriers were recultured, and 12 of them, or 40%, were found still to harbor the meningococcus. It is therefore evident that 3 consecutive negative cultures at 3-day intervals did not insure the permanent clearing of the nasopharynx. Either a longer interval or more negative cultures, or both, should be required. We are of the opinion that more cultures rather than a longer interval would produce the best results, say 5 cultures at 4 or 5-day intervals. We also believe that each chronic carrier should be followed after his release by a "register" similar to that given to malaria patients in the army, so that cultures could be taken at definite intervals, or whenever the carrier entered a new command.

WEATHER CONDITIONS AND THE MENINGOCOCCUS

Most of the cases of meningitis at Camp McClellan during the past winter occurred in January. The highest percentage of carriers as well as the largest number of carriers were also detected during that month. In this region of the country, as everywhere else, the most severe weather of the winter also came during January. We obtained the monthly records of temperature and rainfall for this locality from the U. S. Weather Bureau in Anniston, Ala., and the relation between these weather conditions and the prevalence and activity of the meningococcus is shown in Chart 1.

It will be seen from this chart that during the first 3 months the percentage of carriers among contacts increased more than the number of cases. This may be ascribed to the continued close contact of the troops in their quarters as the weather grew colder. The organism was gradually being passed from one man to the next. During these months the rainfall averaged between 1 and 2 inches per month, and much outdoor training was possible. In January, however, with but slight decrease in the mean temperature, there was an increase of rainfall to over 8 inches, and a coincident marked increase in the number of cases of meningitis and of the percentage of carriers. Then with the return of fair, warm weather early in February, both cases and carriers again fell to a very low number. Apparently the close contact of the troops in tents and mess halls during the inclement weather was an important factor in the rapid spread of the meningococcus. This undoubtedly applies to the spread of all other respiratory infections as well.

CULTURING OF MEN FROM FURLOUGH

All men returning from furlough since Christmas have had naso-pharyngeal cultures taken for meningococcus. This work was done on the assumption that these men might pick up meningococci on trains or at home, and start an epidemic on their return to camp. Of these furlough men, 5,717 were cultured up to March 31. These

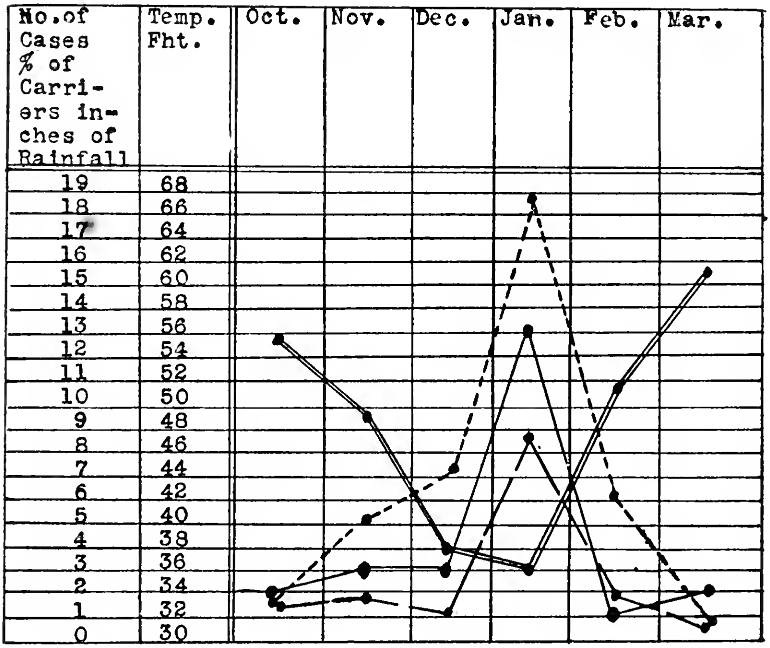


Chart 1.—Relation of weather conditions to the prevalence of the meningococcus and the occurrence of meningitis.

———— = Cases of meningitis.
— — — — = Percentage of contacts* found to be carriers.
—— —— = Rainfall in inches.
═══ Mean temperature — Fahrenheit.

* Contacts are members of companies in which cases of meningitis occurred.

men had all been in camp for several months before their furlough, and were on furlough for about a week. On their return they were placed in an isolation camp until their cultures were reported. Some were from companies in which cases of meningitis had occurred, but most were not. These men, therefore, represent a good average of the whole camp for the occurrence of meningococci. Chart 2 shows

the relation between the percentages of carriers among furlough men and in companies in which cases of meningitis had occurred.

The striking point brought out in this chart is the greatly higher percentage of carriers among contacts than among furlough men during the month of January. This coincides with what has generally been found elsewhere in both civil and military populations.[2] That the percentage of carriers among furlough men was higher in

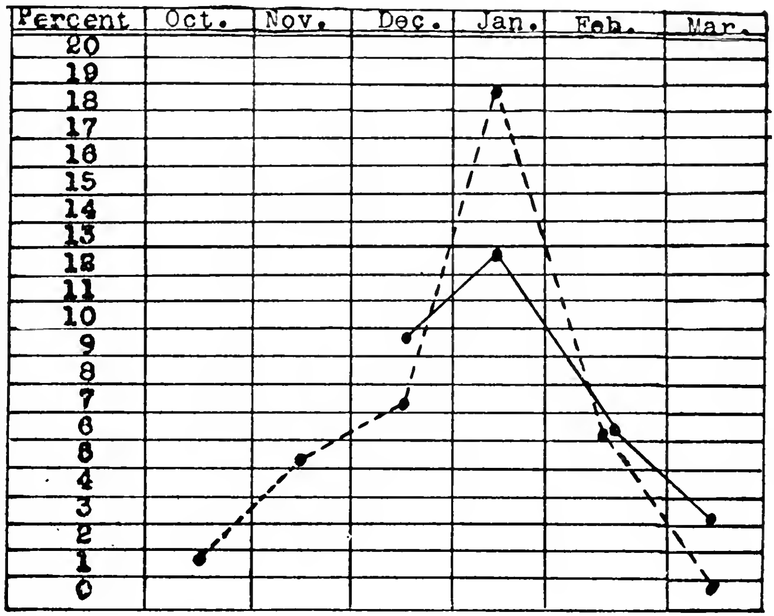


Chart 2.—Carriers among furlough men and contacts.
Solid line = Per cent. of carriers among men returning from furlough.
Dash line = Per cent. of carriers among companies in which meningitis occurred.

December and March than among contacts can be explained. In December all the cultures on furlough men were taken on the last 2 days of the month, that is, very close to the high-rate month of January. The contacts, on the other hand, were cultured at intervals during December. In March one of the contact companies cultured was a troop of mounted military police, who are never all in their quarters at the same time, and in which a small percentage of carriers would therefore be expected. No carriers were found in this company.

[2] Medical Research Committee (English), Special Report Series No. 3.

One purpose which the culturing of the men returning from furlough accomplished, however, was that it definitely freed them from the suspicion of being the cause of the sudden increase in the number of cases of meningitis in January. Had they not been cultured, they would undoubtedly have been suspected of being the source of these infections.

One other point of interest in our work deserves mention. In only one instance did a second case of meningitis occur in a company in which one case had already occurred and the carriers had been separated.* This one instance occurred in April, 3 months after the company was cultured. The first case was caused by a para type of meningococcus, the second by an intermediate B. Neither did a case of meningitis occur among any of the men isolated as carriers. In other cantonments, where definite epidemics of meningitis have occurred during the past winter, cases have appeared among men already isolated as carriers. This forces us to the conclusion that our apparently good results have been due not so much to the large number of carriers isolated as to the fact that our cases were probably a series of sporadic infections rather than an epidemic of meningitis.

CONCLUSIONS

1. The use of a good polyvalent antimeningococcus serum together with 4 types of monovalent serum for agglutination tests, detects more accurately all types of meningococci, in routine work on a large scale, than does either polyvalent or monovalent serum used alone.

2. Most of the meningococcus carriers in a company in which a case of meningitis occurs, harbor an organism of the same type as that causing the case of meningitis.

3. It is usually possible to find a chronic meningococcus carrier in a company where a case of meningitis occurs.

4. Three consecutive negative cultures taken at 3-day intervals does not insure permanent freedom of a carrier from the meningococcus. At least 5 negative cultures should be required, and the carrier cultured at intervals after release.

5. The meningitis at Camp McClellan during the past winter is considered to have been a series of sporadic cases, not an epidemic.

* Cases 16 and 17 (Table 4) both came from the same company, but both occurred before the company was cultured.

THE DIAGNOSIS OF EPIDEMIC MENINGITIS AND THE CONTROL OF ITS TREATMENT BY RAPID BACTERIOLOGIC AND SEROLOGIC METHODS

M. B. Cohen, (West Salem, Ohio)

AND

J. S. Fleming, (Memphis, Tenn.)

First Lieutenants, M. R. C., U. S. Army

From the Laboratory of Evacuation Hospital No. 14 and the Bacteriologic Department of the Base Hospital, Camp Jackson, S. C.

There is no acute disease which requires more acumen for its diagnosis and treatment than epidemic meningitis. There are many abortive cases and many cases of undoubted meningococcus sepsis which can be diagnosed before the advent of meningitis. As success in treatment depends on early diagnosis, it is imperative that the laboratory be called on to aid at the earliest possible moment, as the final diagnosis is always made in the laboratory. To give proper aid the bacteriologist must have a well equipped laboratory and be familiar with rapid methods of culture of the meningococcus.

This organism has always been described as an aerobic gram-negative diplococcus and until recently only aerobic methods have been used for its isolation and cultivation. A number of special mediums have been advocated; their number attest to the difficulty in growing many strains. Attention has been called to the importance of the reaction of the medium and to the fact that the meningococcus will not usually grow when the reaction is over plus 0.5 acid to phenolphthalein.

All these painstaking methods are the result of the attempt to cultivate the meningococcus aerobically, as all have assumed it to be an obligate aerobe. This assumption is incorrect.

Many have studied aerobic and anaerobic organisms and the corresponding methods of cultures, but only a few have investigated the oxygen requirements of organisms which lie between the aerobic and anaerobic ones, and are usually known as facultative. There is a group of organisms which require for their optimum growth an atmosphere in which the percentage of oxygen is slightly less than that of air. The members of this group are usually spoken of as partial tension organisms, and the meningococcus belongs to this group.[1]

Received for publication Aug. 2, 1918.

[1] Cohen, M. B., and Markle, L.: Jour. Am. Med. Assn., 1915, 67, p. 1302; Cohen, M. B.: Ibid., 1918, 70, p. 1999.

When this basic fact is recognized, meningococcus culture becomes almost as simple as staphylococcus culture. One can isolate it on any enriched medium such as blood agar, the reaction of which may be as high as plus 1.0 acid to phenolphthalein.

Originally, partial tension cultures were made by attaching a slant containing the meningococcus material to a freshly inoculated agar slant of B. subtilis. It was assumed that the rapidly growing B. subtilis used up sufficient oxygen to make the gaseous medium of the proper oxygen tension for the meningococcus growth. That this is actually the case is shown by a series of experiments recently carried out: Several meningococcus strains were isolated from spinal fluids by the subtilis method. The cultures were emulsified in broth and a loopful of each was planted on each of several blood agar slants of the same batch of medium. One slant of each strain was incubated aerobically, one was connected with B. subtilis, and the remainder were placed in jars which contained 10, 20, 30, 50 and 75% Co_2, the rest being in all cases air. All cultures were incubated for 24 hours at 37 C. and then examined. The following is a typical record:

June 20: Culture A isolated from spinal fluid by subtilis method. Standard loop of broth emulsion planted on 7 tubes of human blood agar with a reaction of + 0.2 and incubated at 37 C.

June 21: Tube 1, aerobic: no growth.
Tube 2, subtilis: rich growth.
Tube 3, 10% CO_2: very rich growth.
Tube 4, 20% CO_2: very rich growth.
Tube 5, 30% CO_2: good growth.
Tube 6, 50% CO_2: scant growth.
Tube 7, 75% CO_2: several small colonies.

From a number of experiments it was determined that the optimum gas mixture varied from 10-30% CO_2 with air, depending on the strain of the organism. As growth in 10% CO_2 was always as good as in any greater concentration, and always better than at normal oxygen pressure, a mixture of 10% CO_2 and 90% air was adopted as a routine for all meningococcus work.

These CO_2 air mixtures were prepared as follows:

Jars of known capacity which could be tightly sealed were used to hold the cultures. The CO_2 generated from weighed amounts of Na_2CO_3 by the action of sulphuric acid. By knowing the volume of the container and the per cent. of CO_2 desired one determined the amount of CO_2 needed. The exact amount of Na_2CO_3 necessary to produce this volume of gas was determined in the following manner: According to a well known law of physical chemistry (Gay Lussac) the molecular weight of any substance expressed in gm., occupies in the gaseous state at normal temperature and pressure 22.3 liters. As the molecular weight of Na_2CO_3 is 106, 106 gm. will produce 22.3 liters of CO_2. The amount necessary to produce 10% of any known volume

is then easily figured. The variations in the amount of gas formed due to differences in temperature and pressure were ignored as the percentage of air from these factors is too small to be of any significance in an experiment of this kind.

Herrick[2] calls attention to the fact that epidemic meningitis begins as a meningococcus sepsis, and that for a period from a few hours to many days there is no definite localization in the meninges. It is imperative to make a diagnosis at this stage of the disease as by early massive serum treatment it may be possible to prevent the onset of meningitis or at least to render the disease less severe. During this stage the spinal fluid is usually clear and meningococci may or may not be found in direct smears or by the usual method of culture. In 1916 Cohen and Markle reported 15 positive cultures by the subtilis method from clear spinal fluid. Four positive cultures were obtained from the same series aerobically, but direct smears were constantly negative. Partial tension cultures should always be made from such clear fluids. It is of the utmost importance to make blood cultures also. From 10-20 c c of blood are removed from a vein and are placed in 2 flasks of neutral dextrose-infusion broth. These flasks are incubated in an atmosphere of 10% CO_2. Baeslack[3] and associates found meningococci in the blood in 36% of early cases. This percentage can probably be increased by the method described in the foregoing.

In the cases with well developed meningitis the meningococcus can be usually demonstrated in direct smears from the spinal fluid. To isolate it the spinal fluid is centrifuged and a loopful of the sediment is planted on each of 4 or 5 tubes of human blood agar or several loopfuls are spread on the surface of a blood-agar plate. These cultures are then placed in a jar, the proper amount of Na_2Co_3 (4.7 gm. for each liter of CO_2 desired) is placed in a glass in the bottom of the jar and 10% sulphuric acid is added. The jar is tightly sealed as soon as the reaction is complete. Cultures made in the morning will be found to have grown profusely by the next morning. It has been found possible to secure sufficient material from spinal fluid with 24 hours for agglutination reactions with various commercial serums to determine which serum is more suitable for the treatment of the case in question.

The following method of agglutinating has proved satisfactory. As many rows of 7 small test tubes are placed in the rack as there are strains to agglutinate. The first tube of each row is numbered with the number of

[2] Arch. Int. Med., 1918, 21, p. 541.
[3] Jour. Am. Med. Assn., 1918, 70, p. 684.

the corresponding slant. To each of the numbered tubes 2 c c of salt solution is added; 0.8 c c to the last tube, the salt solution control. In the second row place 0.8 c c of a 1:80 dilution of diagnostic polyvalent meningococcic serum. In the third row place 0.8 c c of a 1:80 dilution of a diagnostic normal meningococcic serum. In the fourth row place 0.8 c c of a 1:80 dilution of an intermediate diagnostic serum. In the fifth row place 0.8 c c of a 1:80 dilution of a diagnostic parameningococcic serum. In the sixth row place 0.8 c c of a 1:40 dilution of horse serum. A 1½ mm. loopful of the suspected organism is then scraped off a slant and emulsified in the tube containing 2 c c of salt solution. To each of these diluted serums are added 0.2 c c of the bacterial emulsion, the tubes are shaken and placed in the 56 C. incubator; readings are made in 12-24 hours. By using this method it is possible to secure a type determination without subculturing. Original slants may be subcultured after agglutination has proven it to be a meningococcus.

TABLE 1.—MENINGITIS CASES, CONVALESCENTS, CONTACTS AND NONCONTACTS

Number Throat Cultures Taken	Positive in Air		Positive in CO_2	
	Number	Percentage	Number	Percentage
17	3	17.7	10	59.0
53	2	3.7	9	16.0
32	0	0.0	4	12.4
36	0	0.0	3	8.3
70	1	1.4	9	12.7
12	0	0.0	0	0.0
35	0	0.0	11	31.3
120	0	0.0	4	3.3
37	0	0.0	1	2.7
88	1	1.1	3	3.4
500	7	1.4	54	10.8

The following method has been used for carrier work:

A straight wire possessing moderate elasticity, about 10 inches long with cotton applicator at tip is used in preference to the West tube. These wire swabs were made up in lots of 8 each, placed in test tubes and sterilized. When ready for use one is withdrawn, bent to an angle of 45° about 1½ inches from the tip by using the mouth of the test tube. The wire is then bent once again to a right angle 3 inches from the opposite end of the cotton applicator. This affords a handle and allows the swab to be used with greater dexterity. Cultures are secured by passing the swab into the nasopharynx by way of the mouth. Inoculations are made on one-half of each of 2 blood-agar plates, and one plate is incubated in the usual manner; the other at partial oxygen tension. The medium used is composed of 5% defibrinated human blood, not laked, 1% dextrose in 2% agar, and reaction plus 0.3. The plates are incubated at 37 C. over night and examined the next morning. This is an approximate incubation period of 18 hours. Plates which show suspicious colonies are separated for picking, the others discarded. Quite frequently the aerobic plates may fail to disclose colonies that resemble meningococcus colonies whereas in the duplicate plate grown in CO_2 they appear quite numerous. In the large majority of plates it has been evident that the colonies grown in CO_2 were more luxuriant and vigorous than the aerobic

ones. Suspicious colonies are transferred to blood-agar slants and, after incubation, examined by gram stain and agglutination. Cultures originally grown in CO_2 have been carried in that atmosphere throughout.

Table 1 contrasts the percentage of carriers detected by the new and by the old methods.

Cultures of the last 15 spinal fluids showing gram-negative diplococci microscopically gave positive cultures in 13 of the cases, all cultured in CO_2.

SUMMARY

Attention is again called to the fact that the meningococcus is a microaerophil.

The optimum reduction in oxygen tension for meningococcus cultures has been measured.

Simple, rapid and accurate methods for the isolation and cultivation of the meningococcus have been described.

Meningococci grow best in an atmosphere composed of approximately 10% CO_2 and 90% air.

In 500 nasopharyngeal cultures 10.8% carriers have been found by CO_2, and 1.4% by the usual method.

CARBON DIOXID IN THE PRIMARY CULTIVATION OF THE GONOCOCCUS

Charles W. Chapin

From the Laboratory of the U. S. Immigration Station, Ellis Island, N. Y., and the Laboratory of the U. S. Marine Hospital, Stapleton, N. Y.

In a general investigation of the cultural requirements of the gonococcus it seemed well to test the influence of carbon dioxid at pressures approximating its tension in living tissue, that is, from 5-7 cm. Hg.

For the purpose of this work a gram-negative coccus isolated from the pus of a case of gonorrhea in the male has usually been deemed a gonococcus without further attempt at specific identification. Slight irregularity of contour and more or less variation in size and staining intensity of the individuals, and the transparency or inconspicuousness of the young colonies have been regularly observed characteristics.

For the experiment reported in the table a meat infusion was prepared in the usual way and heated to remove coagulable proteid. To this were added urine, 20%, peptone 0.5%, and a normal dilution of sulphuric acid 1.2%. This mixture was divided in 5 flasks to which were added different percentages of a normal solution of sodium hydroxid as shown in the table, and the reaction readings were taken at this stage of the preparation. The finished medium contained in addition, agar, q. s., pea flour 0.5%, dextrose 0.5%, and egg yolk 5%, the yolk being added to the cooled medium just before pouring the plates. Four plates were poured from each flask that the entire series might be duplicated in each of 2 chambers. On the surface of these plates was spread the slightly diluted gonorrheal pus.

The plates were covered with glass covers and placed in two stacks in a pan; each stack was covered by an inverted mouse jar and a little water was poured in the pan to seal the chambers. The carbon dioxid was prepared by treating sodium bicarbonate with sulphuric acid, and collected over water to which a little sodium bicarbonate had been added. To the atmosphere of one of the jars was added this carbon dioxid, approximately 10%, while the unaltered atmosphere of the other jar served as a control.

After "control" and "carbon dioxid" in the table are shown the readings of the plates on the 2nd day, the best plates from the standpoint of number of gonococcus colonies being given an arbitrary rating of 10, obviously inferior plates some lesser numeral, while negative plates are shown by the minus sign.

It appears from a glance at the table that the test plates have been successful at a wide range of reactions while the results in the control series are very inferior. Considering that carbon dioxid forms an acid

Received for publication April 13, 1918.

on going into solution it might be expected to make an alkaline medium more favorable, acid medium less so, but from the table it appears to have improved the cultural conditions even with highly acid mediums.

This table is a selection from the records of several somewhat similar experiments with mediums containing raw proteid (egg yolk, ascitic fluid, calf serum). The medium and other technical details have been varied in numerous ways, and the pus has been obtained from several different patients during the course of the experiments. The results have been consistent as to the main issue though it has occurred that the control plates have been fairly successful at a limited range of reactions, thus obscuring comparison at the acid end of the series.

TABLE 1

RESULTS OF EXPERIMENTS TO SHOW THE VALUE OF CO_2 IN THE CULTIVATION OF THE GONOCOCCUS

Normal NaOH, per cent	0	1.2	2.3	3.7	4.7
Reaction	3.4	2.6	1.8	0.5	alk.
Control	— —	1 1	1 2	— —	— —
Carbon dioxid	3 3	10 10	10 10	10 8	10 10

There remains to be considered the theoretical possibility that carbon dioxid might improve even an acid medium by its independent effect on the reaction of the raw proteid, which may not have assimilated with the reaction of the rest of the medium at the temperature. My experiments on this point have shown distinct evidence that the carbon dioxid improves such mediums by some effect on the raw proteid constituent, though the data on this point are still insufficient.

Wherry and Oliver[1] have shown that the gonococcus grows best at an oxygen pressure below that of the air, such condition being obtained in most of their experiments by the atmospheric activity of B. subtilis. It would seem that the general principle of their technic might easily be adapted to enrich the atmosphere with carbon dioxid at the same time. A lighted candle left in the jar beside the plates at the time of sealing the chamber, I have found a convenient means of establishing a highly favorable atmosphere.

To summarize, an atmosphere rich in carbon dioxid has been found to facilitate the primary cultivation of the gonococcus on mediums containing raw proteid.

[1] Jour. Infect. Dis., 1916, 19, p. 288.

A COMPARISON OF METHODS FOR THE EXAMINATION OF WATER AT FILTRATION PLANTS

John F. Norton

From the Department of Hygiene and Bacteriology, University of Chicago

It is customary to compare water purification plants on the basis of bacteriologic examinations of raw and treated waters, although the raw water supplies are varied in character and filters of many kinds operated under diverse conditions. Two sorts of results are used: (a) the percentage removal of bacteria during the purifying process as estimated by the ordinary plating method or by determinations of some specific organism as B. coli; and (b) the bacterial character of the treated water regardless of its original micro-organic content. Any comparison by percentage removal is unsatisfactory because the results so largely depend on the number of bacteria in the raw water. It is relatively easy to obtain a 99% "purification" on a water containing 20,000 organisms per c c, leaving 200 still in the filtered supply, and correspondingly difficult to obtain a count of 2 from a raw water containing 200 bacteria per c c — a condition necessary to attain in order to have the same percentage reduction.

But whatever method is chosen for reporting filtration results either for comparing different system of purification or for controlling the process in a single plant, this method is dependent on the procedures in use for obtaining the bacteriologic data. There is great diversity of opinion among bacteriologists as to the most reliable procedures. Although there exists a "Standard Methods of Water Analysis," those in charge of water-works laboratories believe that it is often wise to deviate from these suggested standards.

In order to obtain some definite information concerning the bacteriologic technic in vogue in collecting data on filter plant operation, a series of questions was sent to a selected list of cities where purification works had been in operation for some years. Twenty-four satisfactory replies were received. I am indebted for the material here discussed to those so kindly responding to my request for information. The list contains plants of varying size and type, and includes 6 slow sand filters, 17 rapid filters and one in which coagulation and sedimentation are used but no filtration process. In 6 hypochlorite of lime

Received for publication April 18, 1918.

is applied to the filtered water, 13 utilize liquid chlorin and one uses hypochlorite and ammonia. In 4 no disinfectant is used. The plants are widely distributed over the country and although fewer in number than desired, represent current American practice as nearly as possible.

An examination of the data received has revealed some interesting conditions which are here reported. Perhaps the most striking is that no two of the 24 laboratories use procedures for their bacterial water examinations which are identical in every respect. Certainly comparisons of analytical data under such a state of affairs is, to say the least, unfortunate.

For a detailed study the data may be grouped under 3 headings: (1) total counts on gelatin; (2) total counts on agar, and (3) tests for the presence of organisms of the colon bacillus type. To this has been added another section (4) discussing the relation of the gelatin and agar counts. In only one of the laboratories reporting is the direct microscopic method used, and here only on the raw water as supplementary to mediums.

1. GELATIN COUNTS

Of the 24 laboratories, 15 (62.5% *) employ gelatin for a total count at 20°. In one case agar medium is also used at this temperature and the results reported in the annual statements in preference to the gelatin count. In all but one instance the count is made after 48 hours at 20 C., and in the one exception the time is reported as "48 to 72 hours." The gelatin medium, as far as could be ascertained, is made to conform closely to that suggested in "Standard Methods."

In 12 of the above mentioned 15 laboratories counts are also made on agar at some higher temperature, but in two of these cases no results are reported, so that for practical statistical purposes only 10 laboratories (41.5% of the total) use both gelatin and agar at different temperatures to obtain reportable results of the number of bacteria in the raw and treated waters.

In general, the methods involving the use of a gelatin medium are fairly consistent and the results correspondingly comparable.

2. AGAR COUNTS

Twenty-one laboratories use agar at some temperature for a bacterial count, and with one exception both raw and treated water are examined. The following table shows the different periods of time allowed to elapse and the corresponding temperatures employed.

* The total number of cases is too small to allow arguments on a percentage basis. This is calculated in only a few instances and the figures used with reserve.

TABLE 1

AGAR COUNTS

Time in Hours	Temperature	Number
24	37C*	11
24 and 48	37	1
48	37	1
48 and 24	20 and 37	2
48 and 24	22 and 37	1
48	22	1
72	20	1
72	30	1
48	40	1
24 to 48	38	1
Total		21

Percentage using agar, 87.5%; percentage using agar, 24 hours at 37 C., 58.5%.
* Temperatures reported as 37 or 37.5 are all recorded as 37 C.

As Table 1 shows, agar counts are comparable from only a little over half of the laboratories, and only when the results are obtained from the 37° plates. There is certainly no use in comparing figures from plates incubated 72 hours at 20° with those incubated 48 hours at 40°.

The composition of the agar appears to be fairly uniform, although with the several varieties of peptone now on the market there is some doubt as to this. Nineteen laboratories follow the procedures suggested in "Standard Method," one uses litmus-lactose agar and one adds 2% of peptone instead of the usual 1%.

The weight of opinion is quite strongly for agar counts after 24 hours at 37 C. Fourteen of the 24 laboratories use this method, while 9 of these use also the 48 hour count on gelatin at 20° and 2 use agar at 20°.

3. ORGANISMS OF THE TYPE B. COLI

Organisms of the type of B. coli are used generally as indicators of the sanitary quality of water supplies. A study of the procedures used in 23 laboratories for their detection discloses a great variety of practices. The different mediums used for the primary inoculation, together with the corresponding number of laboratories, is shown in Table 2.

TABLE 2

MEDIUMS FOR PRIMARY INOCULATIONS

Lactose broth	13
Lactose bile	8
Lactose and dextrose broths	1
Lactose agar and dextrose broth	1
Total	23

From Table 2 it is seen that 56.5% use lactose broth and 34.8% utilize a bile medium, although it has been shown by Jordan[1] and others that bile inhibits the growth of the colon bacillus and the medium has been dropped from "Standard Methods." In 2 of the 23 instances lactose-litmus-agar plates are used to check the broth results, and in one laboratory neutral red agar is used.

In 9 cases (about 40%) gas formation, in amounts of 10% or more, in the sugar medium after 24 or 48 hours is regarded as sufficient evidence of the presence of B. coli in the water, the actual numbers being figured from the number of tubes containing gas and the amount of water used. This is, of course, the so-called presumptive test. In 6 of the laboratories using this test lactose bile is used for the primary inoculation (2 using litmus-lactose-agar plates as a check), and the other 3 start with lactose broth (1 using neutral red agar plates as a check). In addition to the foregoing 9, in 3 laboratories the presumptive test is used on the raw water only but in conjunction with confirmatory tests on samples of treated water. Since it is well known that the presumptive test is likely to give high colon counts this procedure tends to give an exaggerated idea of the efficiency of the purification process. The argument used in favor of the presumptive test is "that previous experience has shown a large percentage of the tubes containing gas will give positive confirmatory tests."

The lactose broth used for the primary inoculation does not vary any more than results from the manufacture of the ingredients by different firms, but the lactose bile varies a great deal in respect to the source of the bile. In some laboratories fresh bile is used and in others dried preparations of various makes are utilized.

In making confirmatory tests for colon bacilli from the fermented broths 2 mediums are in use for the first isolation of organisms — Endo medium and lactose litmus agar.* Of the 14 laboratories in which confirmatory tests are used, 8 use Endo plates or streaks and 4 the lactose litmus agar. In one instance both mediums are used and in the other both are used but not on the same sample of water. The weight of opinion is decidedly in favor of Endo medium for this secondary inoculation.

Final diagnosis for colon bacilli by the appearance of colonies on Endo or lactose litmus agar is made in two instances, although in one

[1] Jour. Infect. Dis., 1913, 12, p. 326.

* In one case azolitmin is used. Since the reaction is the same as with litmus there is no reason to regard the medium as distinct for the purpose of isolating colon bacilli.

a doubtful result leads to transfers to test fermentation in dextrose and lactose broths.

In 3 cases the final test consists in the inoculation of lactose broth with organisms from characteristic colonies on Endo medium while in 4 more, colonies from Endo or lactose litmus agar are not only tested for lactose fermentation but are also examined for morphological characteristics after transferring to agar slants.[2]

This leaves 5 laboratories which favor further biochemical reactions for identification of colon bacilli. From one of these no statement was obtained concerning the exact procedure so that Table 3 shows the variety of tests employed in the other 4 cases and the number of times each test occurs. The only test on which there is entire agreement is that for gelatin liquefaction.

TABLE 3

FURTHER TESTS FOR B. COLI

Gelatin liquefaction	4
Indol production	3
Lactose fermentation	2
Nitrate reduction	2
Litmus milk reaction	1
Dextrose fermentation	1
Voges-Proskauer test	1
Methyl-red test	1

To summarize: only 4 of the 23 laboratories follow the so-called "completed test" as suggested in "Standard Methods" (characteristic colonies on Endo, formation of gas in lactose broth and demonstration of nonspore-forming organisms from an agar slant), 5 define colon bacilli in a more rigid manner than this, while the remaining 14 utilize a more liberal interpretation of the characteristics of that group. It is obvious that no proper comparisons of water supplies or of filter efficiencies should be made on such uncertain analytical data.

RELATION BETWEEN GELATIN AND AGAR COUNT

The relative value of agar and gelatin for water examinations has been a matter of some dispute. Those in favor of gelatin (20°) argue that the greater growth gives a more accurate estimate of filtration efficiencies while those preferring agar (37°) insist that it is more important to obtain an estimate of the number of organisms growing at body temperature and which are presumably of sewage origin.

[2] Am. Jour. Pub. Health, 1917, 7, p. 1050, gives a complete description of methods used in one laboratory.

There is an impression that the ratio of the gelatin to the agar count is about 10:1. In the replies to the questions on which this paper is based there is some evidence bearing directly on this point.* In Table 4 the ratio of gelatin counts (48 hours at 20°) to agar counts (24 hours at 37°) has been computed for raw and filtered waters from the data supplied by 5 filter plants. The average monthly counts for a year are combined to give an average yearly count and the ratio of gelatin to agar determined. In order to give an idea of the variations in this ratio and also of the actual numerical value of the counts, the highest and lowest found as monthly averages and the highest and lowest ratios based on these monthly average counts during the same years have been computed and are recorded in Table 5.

TABLE 4

RELATION OF GELATIN TO AGAR COUNTS. YEARLY DATA

	Yearly Average Count				Ratio Gelatin to Agar	
	Raw		Filtered			
	Gelatin	Agar	Gelatin	Agar	Raw	Filtered
1	1,770	580	36	9	3.3	4.0
2	58,300	5,200	149	10	11.1	14.9
3	5,880	388	10.7	4.7	15.1	2.3
4	4,872	1,236	46	56	3.9	0.8
5	36,000	650	13	3	55.5	4.3

TABLE 5

RELATION OF GELATIN TO AGAR COUNTS. MONTHLY DATA

	Monthly Average Count				Ratio Gelatin to Agar	
	Raw		Filtered			
	Gelatin	Agar	Gelatin	Agar	Raw	Filtered
1. High	4,100	1,546	97	15	10.8	12.1
Low	535	121	16	4	1.3	2.3
2. High	2,486,000	15,000	530	16	103.4	86.0
Low	11,200	6,000	14	5	1.2	1.1
3. High	12,700	1,110	44	8	53.0	11.0
Low	280	55	3	3	0.8	0.6
4. High	13,225	2,613	189	206	9.1	7.0
Low	511	362	6	2	0.9	0.2
5. High	72,000	4,400	35	7	338.0	19.0
Low	11,000	210	4	0	5.6	2.0*

* Omitting ratios involving 0 in the denominator.
Note: The ratios as given are those actually obtained for any one month and therefore have no relation directly to the counts given in the table.

An examination of Tables 4 and 5 shows no relation whatever to exist between the gelatin count at 20° and the agar count at 37°. There is, however, a tendency for the ratios to be lower in the filtered than

* See also Tanner, Univ. of Illinois Bull., Water Survey Series, 1916, 12, p. 242.

in the raw water, which indicates a larger relative removal of organisms growing at 20° compared to those growing at 37°, but further than this no definite statement can be made. There is no evidence in these figures that a 10: 1 ratio is maintained.

SUMMARY

Statistics concerning the methods of water examination in connection with 24 purification plants in this country show that in no two instances are these methods exactly alike.

About two-thirds of the laboratories use gelatin as the medium for obtaining a total count at 20 C. The procedures with this medium are fairly consistent.

About seven-eighths of the laboratories use an agar medium for counts at some temperature, while 60% conform to a 24-hour count at 37 C. The composition of the medium is as consistent as possible with ingredients from varying sources.

Many differences are found in the methods for the detection of organisms of the type B. coli. About 60% of the laboratories use lactose broth and most of the remainder use lactose bile. In somewhat less than half of the instances the presumptive test only is made while the others use a great variety of confirmatory tests.

No definite relation is maintained between gelatin and agar counts.

In order to be comparable, data of water examinations must be collected under identical conditions. Since no uniformity in methods exists among the laboratories in this country, bacteriologic data of water supplies and water purification processes are not amenable to critical comparisons.

WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM TWO YEARS OLD

E. H. Ruediger

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

In a previous report[1] I showed that glycerolated human serum a year old was still suitable for the Wassermann reaction, and that the results obtained with it were nearly identical with the results obtained with it while fresh. The present report deals with the same serums tested approximately two years after they had been obtained from the patients.

The serums were heated to about 55.5 C. for 30 minutes before they were mixed with glycerol; the others were discarded because they had become anticomplementary. Some of the serums which had been heated before they were mixed with glycerol were discarded because the tubes were not stoppered tightly enough to prevent evaporation.

The method of testing was identical with that described a year ago and needs no further description at this time.

The serums were obtained in the latter part of 1915 and early in 1916. The serum was drawn off the clot on the day after the bleeding, and each serum was divided into two portions, A and B. Portion A, unglycerolated, was tested at once. Portion B was heated to about 55.5 C. for 30 minutes, mixed with an equal volume of sterilized, pure glycerol, and kept at room temperature in a cork-stoppered test tube. On May 5-6, 1918, Portion B of each serum was tested and the result was compared with that given by Portion A.

The results are shown in the table. The results obtained with Portions B were almost identical with the results obtained with Portions A about two years before. Serum 30 had become strongly anticomplementary and all the other serums had become moderately anticomplementary. On heating the serums to 56 C. for 30 minutes the anticomplementary property disappeared.

SUMMARY

Glycerolated serum two years old gave results almost identical with those given by the fresh serum.

Received for publication May 2, 1918.

[1] Ruediger, E. H.: Jour. Infect. Dis., 1917, 21, p. 502.

TABLE 1

WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM TWO YEARS OLD

No.	Date of Bleeding	Portions: A, Nonglycerolated; B, Glycerolated	Heated	Reheated	Tested	Amboceptor per Tube Unit	Readings* Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Result
1	12/27/15	A	12/28/15		12/28/15	1.0	+	+	tr	+	+	tr	Negative
		B	12/28/15		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	12/28/15	3/5/18	3/5/18	1.25	+	+	tr	+	+	tr	Negative
2	12/27/15	A	12/28/15		12/28/15	1.0	+	+	tr	+	+	tr	Negative
		B	12/28/15		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	12/28/15	3/5/18	3/5/18	1.25	+	+	tr	+	+	tr	Negative
3	12/27/15	A	12/28/15		12/28/15	1.0	+	+	tr	+	+	tr	Negative
		B	12/28/15		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	12/28/15	3/5/18	3/5/18	1.25	+	+	±	+	+	±	Negative
4	12/27/15	A	12/28/15		12/28/15	1.0	+	+	0	+	+	0	Negative
		B	12/28/15		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	12/28/15	3/5/18	3/8/18	1.25	+	+	tr	+	+	tr	Negative
5	12/29/15	A	12/30/15		12/30/15	1.0	+	+	tr	+	+	tr	Negative
		B	12/30/15		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	12/30/15	3/5/18	3/5/18	1.25	+	+	tr	+	+	tr	Negative
6	12/29/15	A	12/30/15		12/30/15	1.0	+	+	tr	+	+	tr	Negative
		B	12/30/15		3/5/18	1.25	+	+	0	+	+	0	Negative
		B	12/30/15	3/5/18	3/5/18	1.25	+	+	±	+	+	±	Negative
7	12/29/15	A	12/30/15		12/30/15	1.0	+	+	tr	+	+	tr	Negative
		B	12/30/15		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	12/30/15	3/5/18	3/5/18	1.25	+	+	0	+	+	0	Negative
8	12/29/15	A	12/30/15		12/30/18	1.0	+	+	tr	+	+	tr	Negative
		B	12/30/15		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	12/30/15	3/5/18	3/5/18	1.25	+	+	tr	+	+	tr	Negative
9	4/5/16	A	4/6/16		4/6/16	1.0	+	+	±	+	+	±	Negative
		B	4/6/16		3/5/18	1.25	+	0	0	+	0	0	Negative
		B	4/6/16	3/5/18	3/5/18	1.25	+	+	0	+	+	0	Negative
10	4/5/16	A	4/6/16		4/6/16	1.0	+	+	±	+	+	±	Negative
		B	4/6/16		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	4/6/16	3/5/18	3/5/18	1.25	+	+	tr	+	+	tr	Negative
11	4/5/16	A	4/6/16		4/6/16	1.0	+	+	±	+	+	±	Negative
		B	4/6/16		3/5/18	1.25	+	+	0	+	+	0	Negative
		B	4/6/16	3/5/18	3/5/18	1.25	+	+	tr	+	+	tr	Negative
12	4/5/16	A	4/6/16		4/6/16	1.0	+	+	±	+	+	±	Negative
		B	4/6/16		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	4/6/16	3/5/18	3/5/18	1.25	+	+	tr	+	+	±	Negative
13	4/7/16	A	4/8/16		4/8/16	1.0	+	+	±	+	+	±	Negative
		B	4/8/16		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	4/8/16	3/5/18	3/5/18	1.25	+	+	±	+	+	±	Negative
14	4/9/16	A	4/10/16		4/10/16	1.0	+	+	±	+	+	±	Negative
		B	4/10/16		3/5/18	1.25	+	±	0	+	±	0	Negative
		B	4/10/16	3/5/18	3/5/18	1.25	+	+	tr	+	+	tr	Negative
15	11/27/15	A	11/28/15		11/28/15	1.0	+	tr	0	+	±	0	Weakly positive
		B	11/28/15		3/5/18	1.25	+	0	0	+	tr	0	Weakly positive
		B	11/28/15	3/5/18	3/5/18	1.25	+	+	0	+	+	tr	Weakly positive
16	4/5/16	A	4/6/16		4/6/16	1.0	+	+	tr	+	+	±	Weakly positive
		B	4/6/16		3/5/18	1.25	+	tr	0	+	+	0	Moderately positive
		B	4/6/16	3/5/18	3/5/18	1.25	+	+	0	+	+	tr	Weakly positive
17	4/5/16	A	4/6/16		4/6/16	1.0	+	+	tr	+	+	±	Weakly positive
		B	4/6/16		3/5/18	1.25	+	±	0	+	+	0	Weakly positive
		B	4/6/16	3/5/18	3/5/18	1.25	+	+	tr	+	+	±	Weakly positive
18	4/10/16	A	4/11/16		4/11/16	1.0	+	+	tr	+	+	±	Weakly positive
		B	4/11/16		3/5/18	1.25	+	tr	0	+	+	0	Moderately positive
		B	4/11/16	3/5/18	3/5/18	1.25	+	+	0	+	+	tr	Weakly positive
19	11/26/15	A	11/27/15		11/27/15	1.0	+	±	0	+	+	0	Weakly positive
		B	11/27/15		3/5/18	1.25	+	tr	0	+	+	0	Moderately positive
		B	11/27/15	3/5/18	3/5/18	1.25	+	+	0	+	+	tr	Weakly positive
20	11/26/15	A	11/27/15		11/27/15	1.0	0	0	0	+	±	0	Strongly positive
		B	11/27/15		3/5/18	1.25	tr	0	0	+	+	0	Strongly positive
		B	11/27/15	3/6/18	3/5/18	1.25	+	0	0	+	+	tr	Strongly positive
21	11/26/15	A	11/27/15		11/27/15	1.0	+	0	0	+	+	tr	Strongly positive
		B	11/27/15		3/6/18	1.25	tr	0	0	+	±	0	Strongly positive
		B	11/27/15	3/6/18	3/6/18	1.25	+	±	0	+	+	±	Strongly positive
22	11/27/15	A	11/28/15		11/28/15	1.0	tr	0	0	+	+	tr	Strongly positive
		B	11/28/15		3/6/18	1.25	0	0	0	+	±	0	Strongly positive
		B	11/28/15	3/6/18	3/6/18	1.25	±	0	0	+	+	tr	Strongly positive

TABLE 1—*Continued*

WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM TWO YEARS OLD

No.	Date of Bleeding	Portions: A, Nonglycerolated; B, Glycerolated	Heated	Reheated	Tested	Amboceptor per Tube Unit	Readings* Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Result
23	11/27/15	A	11/28/15		11/28/15	1.0	+	0	0	+	+	tr	Strongly positive
		B	11/28/15		3/ 6/18	1.25	tr	0	0	+	tr	0	Strongly positive
		B	11/28/15	3/ 6/18	3/ 6/18	1.25	+	tr	0	+	+	tr	Strongly positive
24	11/27/15	A	11/28/15		11/28/15	1.0	tr	0	0	+	+	0	Strongly positive
		B	11/28/15		3/ 6/18	1.25	±	0	0	+	+	0	Strongly positive
		B	11/28/15	3/ 6/18	3/ 6/18	1.25	+	tr	0	+	+	tr	Strongly positive
25	11/30/15	A	12/ 1/15		12/ 1/15	1.0	+	0	0	+	+	tr	Strongly positive
		B	12/ 1/15		3/ 6/18	1.25	0	0	0	+	tr	0	Strongly positive
		B	12/ 1/15	3/ 6/18	3/ 6/18	1.25	+	0	0	+	+	tr	Strongly positive
26	11/30/15	A	12/ 1/15		12/ 1/15	1.0	+	tr	0	+	+	tr	Strongly positive
		B	12/ 1/15		3/ 6/18	1.25	±	0	0	+	±	0	Strongly positive
		B	12/ 1/15	3/ 6/18	3/ 6/18	1.25	+	+	0	+	+	±	Moderately positive
27	4/ 5/16	A	4/ 6/16		4/ 6/16	1.0	+	±	0	+	+	±	Strongly positive
		B	4/ 6/16		3/ 6/18	1.25	tr	0	0	+	tr	0	Strongly positive
		B	4/ 6/16	3/ 6/18	3/ 6/18	1.25	+	tr	0	+	+	tr	Strongly positive
28	4/ 5/16	A	4/ 6/16		4/ 6/16	1.0	+	±	0	+	+	±	Strongly positive
		B	4/ 6/16		3/ 6/18	1.25	+	0	0	+	+	0	Strongly positive
		B	4/ 6/16	3/ 6/18	3/ 6/18	1.25	+	±	0	+	+	±	Strongly positive
29	11/27/15	A	11/28/15		11/28/15	1.0	0	0	0	+	+	tr	Strongly positive
		B	11/28/15		3/ 6/18	1.25	0	0	0	+	±	0	Strongly positive
		B	11/28/15	3/ 6/18	3/ 6/18	1.25	0	0	0	+	+	tr	Strongly positive
30	11/30/15	A	12/ 1/15		12/ 1/15	1.0	0	0	0	+	+	0	Strongly positive
		B	12/ 1/15		3/ 6/18	1.25	0	0	0	0	0	0	Anticomplementary
		B	12/ 1/15	3/ 6/18	3/ 6/18	1.25	0	0	0	+	±	0	Strongly positive

* Explanation: + = complete hemolysis; ± = hemolysis between 50% and 100%; tr (trace) = hemolysis up to 50%; 0 = no hemolysis.

All serums had become somewhat anticomplementary but the anticomplementary property was destroyed by heating to 56 C. for 30 minutes. The result obtained with the Wassermann reaction was not affected by the second heating.

Because of recent modifications in the technic for the Wassermann reaction, and because of the difficulty encountered in keeping serum in cork-stoppered test tubes, there will be no further reports on these serums. A new series has been started in which the serum is kept in hermetically sealed glass ampoules.

FURTHER STUDIES ON BACTERIUM ABORTUS AND RELATED BACTERIA

III. BACTERIUM ABORTUS AND RELATED BACTERIA IN COW'S MILK

Alice C. Evans

From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture, Washington, D. C.

INTRODUCTION

In any consideration of the infectiousness of a food substance, the number of virulent contaminating organisms is an important factor. In the study reported in this paper, the methods were designed to give results as to the numbers as well as kinds of bacteria of the type of Bacterium abortus and related strains found in freshly drawn milk. The methods were practically the same as those employed in an earlier work of which this is a continuation. The data presented in this paper are therefore supplemental to the earlier report.[1]

It was reported in the earlier paper that besides the typical virulent strains of Bact. abortus, other bacteria of similar morphology were eliminated from the udders of many cows whose milk was examined. The history of those cows was not known. Many other samples of milk have been examined from cows whose history is known in order to determine whether there might be any correlation between the presence of organisms similar to Bact. abortus in the milk and the occurrence of abortion. The fact that the serum of a certain small percentage of aborting cows does not react on the typical strains of Bact. abortus suggested the possibility that some of these bacteria may sometimes be responsible for abortion.

The method of obtaining cultures was as follows: The aseptically drawn milk was plated on plain infusion agar and on infusion agar to which 10% of sterile raw cow's blood serum had been added. After 4 or 5 days' incubation at 37 C. a sufficient number of colonies to represent every kind of colony growing on both sets of plates were fished off and inoculated into litmus whole milk. Usually 10-20 colonies were transferred from each set of plates. A thorough study was made of every strain thus obtained which was morphologically similar to Bact. abortus.

Received for publication May 11, 1918.

[1] Jour. Infect. Dis., 1916, 18, p. 437.

The results of the milk examinations are presented in the tables in which 3 types of organisms are considered. Under the heading "Bact. abortus" in the tables there are included only those strains which were agglutinated in high dilutions of Bact. abortus antiserum. Under the heading "Abortus-like bacteria" are included strains which resembled the typical strains in cultural characteristics as well as in morphology, but which were agglutinated only in low dilutions of Bact. abortus antiserum. Under the heading "Bact. lipolyticus" are included the fat decomposing strains described in the earlier publication, and under this heading there are also included strains which resemble Bact. lipolyticus culturally, but which vary slightly in their biochemical reactions.

BACT. ABORTUS AND RELATED BACTERIA IN THE MILK OF COWS WHICH HAVE NOT ABORTED

The numbers of Bact. abortus and related bacteria which were found in the milk of 24 cows which had not aborted is given in Table 1. The cows belonged to a herd in which there had been occasional cases

TABLE 1

BACT. ABORTUS AND RELATED BACTERIA IN THE MILK OF COWS OF HERD A THAT HAVE NOT ABORTED

Cow	Number of Bact. Abortus per C c	Abortus-like Bacteria			Bact. Lipolyticus		
		Number per C c	No. of Strains	Names of Strains	Number per C c	No. of Strains	Names of Strains
1	0	0			0		
4	0	100	1	zj	0		
12	0	0			1,600	2	ze, yu
17	0	0			0		
23	0	0			3,800	1	yb
24	0	80	1	xv	140	1	xy
26	0	0			0		
27	0	0			0		
28	0	0			0		
34	0	0			0		
39	0	0			0		
41	0	0			0		
43	0	0			0		
44	0	30	1	yo	0		
48	0	0			0		
51	0	10	1	yl	0		
52	0	0			0		
53	0	0			0		
100	0	0			960	2	zt, zv
106	0	0			0		
107	0	600	2	zp, zq	300	1	zn
114	0	0			0		
206	0	0			500	1	zo
211	0	600	1	zl	0		

of abortion during the few years previous to the examination of the milk samples. Typical strains of Bact. abortus were never cultivated from the milk of the cows which had not aborted, but abortus-like strains were cultivated from the milk of 6, or 25% of them. Bact. lipolyticus was also cultivated from 25% of the cows. If the 2 columns are considered together, bacteria related to Bact. abortus were culti-

TABLE 2

BACT. ABORTUS AND RELATED BACTERIA IN THE MILK OF COWS THAT HAVE ABORTED AS A RESULT OF NATURAL INFECTION

Herd	Cow	Date Abortion Occurred	Agglutination Tests*: Date	Agglutination Tests*: Reaction of Blood Serum	Date Milk Was Examined	Bact. Abortus per C c	Abortus-like Bacteria: No. per C c	Abortus-like Bacteria: No. of Strains	Abortus-like Bacteria: Names of Strains	Bact. Lipolyticus: No. per C c	Bact. Lipolyticus: No. of Strains	Bact. Lipolyticus: Names of Strains
A	18	7/20/17	11/4/16 10/16/17	— +	4/30/17 9/22/17	0 0	0 0			0 4,500	2	zy, aaa
	19	5/6/15	11/4/16 10/18/17 1/25/18	— — +	4/30/17 9/22/17	0 0	0 0			4,000 2,000	1 1	yq aag
	21	8/11/13	11/15/16 10/18/17 11/27/17	— + +	3/2/17	0	0			1,600	1	ya
	33	6/19/15	11/4/16 10/18/17	— +	3/2/17 9/22/17	0 0	70 0	2	xp, xq	0 0		
	40	8/12/17	11/4/16 10/18/17	— +	9/22/17	0	250	1	aae	0		
	45	11/2/16	11/4/16 10/18/17	+ +	3/2/17 9/22/17	0 0	400 0	4	xh, xj, xm, xw	0 2,000	2	aai, aaj
	46	7/21/17	11/4/16 10/18/17	— +	4/30/17 9/22/17	0 0	0 0			9,600 2,500	2 1	ym, zd aak
	50	10/26/16	11/16/16 10/18/17	+ +	3/2/17	0	60	2	xf, xn	0		
	109	12/2/16	11/4/16 10/18/17 11/27/18	— — —	3/2/17	0	15	1	xs	0		
B	28916	11/6/17	4/28/17 6/4/17 8/29/17 11/6/17	— — — +	11/10/17	0	0			8,000	1	abg
	27723	10/6/17	8/21/16 6/4/17 10/6/17 10/27/17	— — — —	10/10/17 10/27/17	0 0	130 1,600	1 2	aau abc, abd	800 900	2	aas, aat
C	317	12/18/16	10/1/16 1/20/17 1/18/18	+ + † +	5/3/17 10/5/17	0 0	0 0			120 1,800	1 1	zg aav

* The agglutination tests recorded in this table, and also those recorded in Tables 3 and 5, were made by the investigators of the Pathological Division, who kindly gave any information in their records which could be correlated with this study.

† The serum from a sample of mixed milk gave a negative reaction in February, 1917, and the serums from the milk samples taken separately from the 4 quarters all gave a negative reaction in December, 1917.

vated from the milk of 10, or 41.7% of the 24 cows. The names of the strains of bacteria related to Bact. abortus are given in Table 1. The characteristics of these strains are given in Table 4. Their characteristics and their relationship to Bact. abortus will be discussed later.

BACT. ABORTUS AND RELATED BACTERIA IN THE MILK OF COWS WHICH HAVE ABORTED

The number of Bact. abortus and related bacteria found in the milk of 12 cows which have aborted as a result of natural infection are given in Table 2. Nine of the cows belonged to the same herd (Herd A) as the cows considered in Table 1. A study of this table brings out 2 significant facts: (1) although the serum reactions of these cows indi-

TABLE 3

BACT. ABORTUS AND RELATED BACTERIA IN THE MILK OF COWS THAT HAVE ABORTED AS A RESULT OF INOCULATION

Cow	Date of Inoculation	Date of Abortion	Agglutination Tests			Date Milk Was Examined	No. of Bact. Abortus per C c	No. of Abortus-like Bacteria per C c	No. of Bact. Lipolyticus per C c
			Blood Serum	Milk Serum					
				Date	Reaction				
50	Numerous inoculations		Continuously positive			2/16/17 5/ 3/17* 10/ 5/17*	25,000 35,000 25,000	0 0 0	25,000 15,000 25,000
51	Numerous inoculations		Continuously positive			5/ 3/17* 10/ 5/17*	145,000 120,000	0 0	0 0
313	10/14/16	1/15/17	Continuously positive	12/20/17	slight	2/16/17 5/ 3/17* 10/ 5/17*	450 0 0	0 0 0	0 30 000 0
314	10/14/16	3/19/17	Continuously positive	12/20/17	strong	5/ 3/17 10/ 5/17	0 0	0 0	0 0

* The cow was dry. A serum-like material from the udder was plated.

cated that at least 10 of the 12 were infected with Bact. abortus, typical strains were never cultivated from any of these samples of milk, and (2) related bacteria were cultivated from 100% of the cows. Abortus-like strains were cultivated from 6, or 50%, of the 12 cows. The numbers of Bact. lipolyticus per c c of milk were noticeably larger in the case of those cows which had aborted, as compared with those which had not aborted, as shown in Table 1.

The prevalence of Bact. abortus and related bacteria in the udders of cows which have aborted as a result of inoculation is shown in Table 3. All 4 cows were inoculated by investigators of the Patho-

logical Division, Bureau of Animal Industry, for purposes of another experiment. Cows 50 and 51 were inoculated many times with a mixture of several strains of Bact. abortus, with the result that their blood serum agglutinated the antigen in higher dilutions than the serum of naturally infected cows. The serum from these cows was used for the agglutination tests recorded in Table 4. Cows 313 and 314 were inoculated only once with the same strains of Bact. abortus as were used for the inoculation of Cows 50 and 51. Every examination of the milk (or serum-like material from the udder in case the cow was dry) of Cows 50 and 51 showed very great numbers of typical Bact. abortus per c c. One month after Cow 313 had aborted the plates showed 450 Bact. abortus per c c of milk. Three months later, when the cow was dry, they had disappeared from the udder. No Bact. abortus could be found in the milk of Cow 314, 1½ months and 6½ months after abortion. Bact. lipolyticus was cultivated from the udders of 2, or 50% of the 4 cows. The numbers of Bact. lipolyticus in the milk or other material from the udder of Cows 50 and 313 were very high. No abortus-like strains were cultivated from the udders of any of these inoculated cows.

GENERAL CONCLUSIONS IN REGARD TO THE PREVALENCE OF VIRULENT STRAINS OF BACT. ABORTUS IN COW'S MILK

The data presented in Tables 1, 2, and 3 offer some suggestions as to the actual numbers of virulent strains of Bact. abortus in cow's milk. It was not surprising that none of these organisms could be cultivated from the milk of cows that had not aborted, but in view of the work of other investigators it was to be expected that if the cultural methods were adequate Bact. abortus could be demonstrated to be in the milk of the cows which had aborted as a result of natural infection. Schroeder and Cotton,[2] Fabyan,[3] and Fleischner and Meyer,[4] determined the presence of Bact. abortus in cow's milk by inoculating guinea-pigs with the milk. They never isolated the organism directly from the milk, and therefore obtained no information concerning the actual number of organisms present. Zwick and Krage[5] cultivated Bact. abortus from cow's milk by centrifuging the milk and streaking the sediment on agar slopes. This method also failed to give informa-

[2] Twenty-Eighth Ann. Report Bur. An. Ind., Department of Agriculture, 1911, p. 139.
[3] Jour. Med. Research, 1913, 28, p. 85.
[4] Am. Jour. Dis. Child., 1917, 14, p. 157.
[5] Berlin Tier. Wchnschr., 1913, 29, p. 41.

tion as to the actual number of organisms present. Cooledge[6, 7] has adopted the agglutinative reaction of the milk for Bact. abortus as a test indicating the distribution of that organism. Such a test, however, not only fails to give information concerning the number of organisms present, but also, in all probability, it frequently indicates udder infection after it no longer exists. Cooledge[6] states that he could not always find the organism by animal inoculation when agglutinins were demonstrated to be in the milk.

Should the fact that virulent Bact. abortus has not been cultivated from the milk of the naturally infected cows be explained by assuming that the method failed to demonstrate them although they were present? The first growth on artificial mediums of Bact. abortus from pathologic material is known to be somewhat difficult to obtain. But the infusion agar containing 10% of raw cow's blood serum which was used for plating the milk is a good medium for the growth of this organism. It seems probable that if there were large numbers of Bact. abortus in the milk, colonies would have appeared on some of the plates. However that may be, the plating method employed was demonstrated to be adequate for the cultivation of those strains which were used for inoculation, as shown in Table 3. Fabyan[8] noted that strains of Bact. abortus which have become adapted to growth on artificial mediums may be passed through animals and recovered from the lesions more readily than strains cultivated from pathologic material for the first time. The investigators of the Pathological Division report that they have had the same experience. It may be that the reason that Bact. abortus could be cultivated from the milk of the inoculated cows was because those strains had previously become adapted to growth on artificial mediums. At any rate the essential point is that the strains of Bact. abortus used for inoculation could be cultivated from the milk. The heavy infection of the udders of Cows 50 and 51 cannot be compared with natural conditions, for these cows were heavily inoculated again and again. Since Cows 313 and 314 were inoculated with the same strains as Cows 50 and 51, we can assume that if Bact. abortus were present in considerable numbers in their udders it could have been demonstrated as well as in the case of Cows 50 and 51. It was demonstrated in the milk of Cow 313 one month after abortion occurred, but could not be cultivated the 3d and

[6] Jour. Agri. Research, 1916, 5, p. 871.
[7] Ibid., 1917, 37, p. 207.
[8] Ibid., 1912, 26, p. 441.

8th months later, although the blood serum continued to give a positive reaction. Unfortunately no examinations were made of the milk of Cow 314 until about 1½ months after abortion, when Bact. abortus had apparently disappeared from the milk, although in this case also the blood serum gave a positive agglutination reaction. However, Bact. abortus may have been present in such small numbers that it failed to be isolated. An example of this possibility is the presence of Strain yl in the milk of Cow 51 of Herd A (Table 1) in such small numbers that the result of plating showed only 10 per c c. Since a 1 : 10 dilution was the lowest made, the chances are that an organism present in such small numbers would not grow in the plate culture, whereas the inoculation of milk containing 10 virulent Bact. abortus per c c might give a positive result.

The results obtained by the plating of milk samples indicate that virulent strains of Bact. abortus are not continuously eliminated in considerable numbers in the milk of cows which have been infected, although the blood serum may continue to give a positive agglutination reaction. On the other hand, the persistence of great numbers of Bact. abortus in the udders of Cows 50 and 51 may illustrate the possible numbers that a badly infected cow could excrete in her milk during the height of her infection.

THE PREVALENCE OF ABORTUS-LIKE BACTERIA IN COW'S MILK

Under the heading "Abortus-Like Bacteria" in the tables are included all strains with the morphology and cultural characteristics of Bact. abortus, but which were agglutinated only in low dilutions of Bact. abortus antiserum. From some of the samples of milk, strains were obtained of similar morphology which were not agglutinated in the 1 : 20 dilution — the lowest tested dilution of the serum. Usually such strains were distinctly different from Bact. abortus in their biochemical reactions. The cultures which showed no agglutinative reaction were discarded, and are not considered in this paper, for they were not found in the milk in any considerable numbers. In response to the agglutination test the abortus-like strains varied widely from Strains xs, abd, and xq (Table 4) which were agglutinated in the 1 : 160 dilution, to strain xt, which was agglutinated in the 1 : 20 dilution. The biochemical reactions of the abortus-like strains are given in Table 4, where they may be compared with the reactions of a typical virulent strain (aap). It may be observed that strain xt, which was acted on only in the lowest dilution of Bact. abortus antiserum, and

TABLE 4

Characteristics of Bact. Abortus and Related Bacteria

Type of Organism	Strain	Reaction in Litmus Whole Milk	Fermentation of: Dextrose	Fermentation of: Lactose	Fermentation of: Saccharose	Fermentation of: Maltose	Fermentation of: Mannite	Decomposition of: Urea	Decomposition of: Asparagin	Reaction in: Nitrate Broth	Reaction in: Gelatin	Reduction of Hydrogen-ion Concentration in Broth Cultures Recorded in PH Values*	Agglutination Reactions in Bact. Abortus Antiserum: 1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Bact. abortus	aap	alkaline	—	—	—	—	—	+	+	slight	—	0.7	C	C	C	C	C	C	+	0
Abortus-like strains	abd	no change	—	—	—	—	—	—	+	+	—	1.3		++	++	+	0	0		
	xs	no change	slight	—	—	—	—	—	—	—	—	1.0	C	C	++	+	0	0		
	xq	no change	slight	—	—	—	—	—	—	—	—	0.3		+	+	+	0			
	xh, xn	acid in cream layer, alkaline beneath	slight	—	—	—	—	+	+	slight	—	0.9		++	+	0	0			
	xm	alkaline	slight	—	—	—	—	+	+	—	—	1.0		++	+	0	0			
	xw	no change	slight	—	slight	—	—	+	+	—	—	0.8		+	+	0	0			
	xv, aac	alkaline	—	—	—	—	—	+	+	±	—	1.0		++	0	0	0			
	xj, zj	acid in cream layer, alkaline beneath	—	—	—	—	—	—	—	—	—	0.8		+	0	0	0			
	xf, xp	alkaline	slight	—	—	—	—	—	—	—	—	0.5		+	0	0	0			
	abe	alkaline	slight	—	slight	—	—	—	slight	—	—	0.7		+	0	0	0			
	yl		—	—	—	—	—	—	—	—	—	1.0		+	0	0	0			
	aau	alkaline	—	—	—	—	—	+	+	—	+	2.2		+	0	0	0			
	zl	alkaline	—	—	—	—	—	—	+	—	+	1.0		+	0	0	0			
	abe	alkaline	slight	slight	slight	slight	slight	+	slight	—	+	1.0	+	+	0	0	0			
	xt	alkaline	slight	—	—	—	—	+	+	—	—	1.0	+	0	0	0	0			
Atypical strains of Bact. lipolyticus	zd, ze, zn, zy, ys, aaj, aak	acid in cream layer	—	—	—	—	—	—	—	—	—	0.3		+	0	0	0			
	aat	acid in cream layer	slight	—		slight	—	—	—	—	—	0.3								
	xy, xa, yb, yr, aav	acid in cream layer	slight	slight	—	—	—	±	—	—	—	0.0								
	aag	no change	—	—	—	—	—	+	+	—	—	0.0								
	ym, yu, zv, aai	no change	—	—	—	—	—	—	—	—	—	0.0								
Bact. lipolyticus	zg, zo, zt, aaa, aaq, aas, abg	acid in cream layer	—	—	—	—	—	—	—	—	—	0.0								

* The initial hydrogen-ion concentration of the broth was 6.0 PH.

several of the other strains agree very closely in biochemical reactions with the typical virulent strain of Bact. abortus. Some of the strains vary considerably from the type strain in their biochemical reactions. Some fail to produce alkali in milk; some are able to decompose the fat of the cream layer, in which respect they resemble Bact. lipolyticus. There is a tendency for the abortus-like strains to attack the carbohydrates slightly, with dextrose yielding most readily. Urea and asparagin, are decomposed by about one half of the strains. Three of the strains, aau, zl, and abc, are distinctly atypical in their liquefaction of gelatin. All the abortus-like strains reduced the hydrogen-ion concentration of broth cultures, but there was a considerable variation in the extent of reduction. Strain aau, which gave a reduction of the hydrogen-ion concentration equivalent to $2.2P_H$ is distinctly atypical, for it reacts in this respect with 3 times the vigor of the typical strains. It agrees with Bact. bronchisepticus (Table 1 of the 2d paper of this series)[9] in every respect except that it liquefies gelatin very slightly.

Abortus-like strains were isolated from 6 of 24, or 25%, of the cows which had not aborted (Table 1). They were isolated from 6 of 12, or 50%, of the cows that had aborted as a result of natural infection (Table 2). They were never isolated from the contents of the udders of the inoculated cows.

Judged by the figures resulting from the study of the limited number of samples of milk, abortus-like strains occur in the milk of cows which have aborted with twice the frequency with which they occur in the milk of cows which have not aborted.

The criticism has been suggested that since the serum from only 2 cows was used for the agglutination tests, these cows at some time may have been infected with the abortus-like organisms, and for this reason, and not because the organisms are related to Bact. abortus, positive serologic reactions were obtained. But Cows 50 and 51, which supplied the serum for these tests, were found to be particularly free from infection with abortus-like bacteria (Table 3). Furthermore, it would be unreasonable to assume that any 2 cows would during their lifetime harbor so many varieties of abortus-like bacteria as were shown to be agglutinated by the serum of these 2 cows (Table 4).

In only one case were large numbers of abortus-like bacteria found in the milk. From the milk of Cow 27723, of Herd B (Table 2), 1,600 of these organisms were found per c c. There were 2 strains of

[9] Jour. Infect. Dis., 1918, 22, p. 583.

them, but six sevenths of the number were of Strain abd, which was agglutinated in the 1 : 160 dilution of Bact. abortus antiserum (Table 4, Line 2, under "Abortus-like Bacteria"). The finding of a large number of bacteria of an abortus-like organism in the milk of this cow is of especial interest, for she had aborted 3 weeks before it was isolated from her milk, and on the day of abortion her serum reacted negatively to a suspension of a typical strain of Bact. abortus. It is well in this connection to recall the fact that the agglutinating power of the Bact. abortus antiserum used for the tests recorded in Table 4, was much stronger than that of a naturally infected cow, and, therefore, it might show relationships which would not be made evident by the use of the serum of a naturally infected cow.

COMPARISON OF BACT. ABORTUS AND BACT. LIPOLYTICUS

In the earlier publication[1] a description was given of a fat decomposing bacterium found in cow's milk which agreed with Bang's[10] original description of the organism causing contagious abortion. The first paper of this series[11] shows that Bact. lipolyticus is not pathogenic to guinea-pigs. In morphology it is identical with Bact. abortus, but Bact. lipolyticus is gram-positive, whereas Bact. abortus is gram-negative. In Table 4, the biochemical reactions of typical strains of Bact. lipolyticus can be compared with the reactions of a typical virulent strain of Bact. abortus (aap). The 2 types of organisms are alike in their failure to decompose the carbohydrates and mannite, and in their failure to liquefy gelatin; but they differ markedly in their reaction in several kinds of mediums. In whole milk Bact. lipolyticus produces an acidity by decomposing fat, whereas Bact. abortus produces an alkalinity. Bact. lipolyticus does not decompose urea and asparagin, whereas Bact. abortus does. In broth cultures Bact. lipolyticus grows without changing the hydrogen-ion concentration, whereas Bact. abortus reduces it considerably. In cultural characteristics also the two types of organisms differ distinctly. Bact. lipolyticus does not cloud broth cultures, as does Bact. abortus, but it forms dustlike granules which sink to the bottom of the tube. In agar shake cultures, Bact. lipolyticus grows in a "diaphragm" a few mm. beneath the surface of the agar. Usually scattered colonies extend upward from this diaphragm toward the surface, but there is no visible growth beneath the diaphragm. Years of artificial cultivation do not

[10] Ztschr. Thiermedicin, 1897, 1, p. 241.
[11] Jour. Infect. Dis., 1918, 22, p. 576.

alter this relationship of Bact. lipolyticus to the supply of oxygen. Bact. abortus produces a heavy surface growth, but no colonies appear beneath the surface in agar shake cultures of strains which have adapted themselves to artificial cultivation, although freshly isolated strains require a reduced oxygen tension. On agar slopes Bact. lipolyticus produces an exceedingly faint dry growth in separate colonies, whereas Bact. abortus produces an abundant, confluent, moist growth. Bact. lipolyticus is greatly favored by the addition of serum to the agar and on such a medium it produces an abundant granular growth. After becoming accustomed to growth on artificial mediums Bact. abortus grows abundantly without the addition of blood serum, and is not especially favored by it.

Since there is such a marked difference in biochemical reactions and cultural characteristics between Bact. lipolyticus and Bact. abortus, it is a fact worthy of attention that Bang's original description of the organism causing contagious abortion agrees with our cultures of Bact. lipolyticus, and does not agree with our cultures of Bact. abortus. Bang noted that the growth of his organism was confined to a zone a few mm. beneath the surface of the agar, that growth in broth culture was in the form of a fine granular sediment, and that growth was greatly favored by the addition of serum to the agar. It would seem by comparing our strains of virulent Bact. abortus with Bang's original description of his organism that there must be more than one variety of bacterium capable of causing abortion in cattle.

In consideration of the probability that typical strains of Bact. abortus are not responsible for every case of abortion among cattle, the bacteriologic findings in the following case are suggestive. Oct. 6, 1917, Cow 27723 of Herd B (Table 2) aborted, and the investigators of the Pathological Division found that her serum reacted negatively to the agglutination test with a suspension of Bact. abortus. They were unable to cultivate Bact. abortus from the pathologic material. Four days after the abortion occurred they submitted to the writer a sample of the uterine exudate, and a sample of milk from this cow. Of the cultures inoculated with the exudate there appeared innumerable colonies on those plates which were incubated in a closed jar with B. subtilis, and on the serum agar plates incubated in ordinary atmosphere. The colonies did not have the characteristic "dew drop" appearance of Bact. abortus, but were the tiny, opaque, compact colonies characteristic of Bact. lipolyticus. Only 2 or 3 of many

colonies transferred to agar slopes grew, and these were cultures which had been incubated in the closed jar with B. subtilis. The growth was scant, in separate colonies, with a granular sediment in the condensation water. In cultural characteristics, therefore, the organism agreed with Bact. lipolyticus. It agreed conspicuously with Bang's description of his organism in its dependence on B. subtilis to render the atmosphere favorable for its growth. It was an exceedingly delicate organism and died 2 or 3 weeks after isolation, before any further study could be made of it.

Table 2 shows that 2 strains of Bact. lipolyticus were obtained from the milk of this cow, and also 3 strains of atypical Bact. abortus. One of them, abd, which was agglutinated in the 1 : 160 dilution of Bact. abortus antiserum, has already been discussed. Although mindful that the data obtained prove nothing, they are, nevertheless, suggestive in showing that in plate cultures inoculated with the uterine exudate of this cow, which aborted with her serum reacting negatively to the agglutination test with a suspension of Bact. abortus, there appeared innumerable colonies of an organism which differed from our strains of Bact. abortus, but agreed culturally with Bang's original description of the abortion organism; and that the milk of this cow was infected with strains of Bact. lipolyticus which agreed culturally with the strain from the exudate, and also the milk was infected with abortus-like strains, one of which was agglutinated in a 1 : 160 dilution of Bact. abortus antiserum. Certainly the cow was badly infected with abortion-like organisms, but so far as the investigations of the 2 laboratories could indicate, she was not infected with typical Bact. abortus.

In order to compare our strains of Bact. abortus with the abortion organism recognized in Denmark, a strain was obtained from Professor Bang in Nov., 1916. When received, the agar shake culture had a heavy surface growth and a delicate diaphragm a few mm. beneath the surface. At the time of this writing, 16 months after the culture was received, this strain has not become adapted to growth in ordinary atmosphere, but is dependent on a simultaneous growth of B. subtilis in a closed jar to render the atmosphere favorable for its growth. In this respect it differs from our strains of Bact. abortus. But the growth on agar slope resembles that of our strains, and it also resembles our strains in clouding broth medium. It is agglutinated by the same dilutions of Bact. abortus antiserum as our virulent strains.

It could not be compared with our strains with respect to biochemical reactions, for the ammonia given off by B. subtilis was absorbed by the medium, thus masking the changes produced by the Bact. abortus culture. Notwithstanding its continuous dependence on B. subtilis, this strain of Professor Bang resembles our typical strains of Bact. abortus rather than our strains of Bact. lipolyticus.

It is impossible to compare Bact. lipolyticus with Bact. abortus in respect to agglutination reactions, because of the nature of the growth of Bact. lipolyticus. It has been noted that growth in broth cultures appears in the form of a granular sediment. The same granular structure appears also on agar slope cultures. The granules are so compact that vigorous shaking will not break them. On plain agar the growth is too scant to obtain a suspension for the agglutinating test. On serum agar an abundant growth may be obtained, but when passed through filter paper the granules are held back and the filtrate is too clear to serve for the test. It was found that if the suspension was allowed to stand until the largest granules had settled to the bottom, the top could be pipetted off for use in the agglutination test. But such a suspension has so great a tendency to arrange itself in clumps that agglutination in dilutions of the antiserum could never be accurately distinguished.

ATYPICAL STRAINS OF BACT. LIPOLYTICUS

As there are abortus-like bacteria, so there are atypical strains of Bact. lipolyticus. Some of the abortus-like strains vary from the type strain by the possession of properties characteristic of Bact. lipolyticus, and vice versa, some of the atypical strains of bact. lipolyticus vary from the type strain by the possession of properties characteristic of Bact. abortus. The intermediary strains form connecting links between the 2 types, and indicate the relationship between them. Strains xh, xn, xj, and zj (Table 4) are illustrations of abortus-like strains which possess properties characteristic for Bact. lipolyticus. These strains decompose the fat with the formation of acid in the cream layer of litmus whole milk while at the same time they resemble the typical Bact. abortus in rendering alkaline the milk beneath the cream layer. Otherwise these 4 strains could scarcely be distinguished from typical Bact. abortus, except for the fact that they are agglutinated only in the low dilution of 1:40 or 1:80 of the Bact. abortus antiserum.

In the first line of Table 4 under the heading "atypical strains of Bact. lipolyticus" are given the characteristics of a number of strains

which possess the cultural characteristics of the typical Bact. lipolyticus and which vary biochemically from the type strains only in their ability to reduce the hydrogen-ion concentration of broth cultures, a characteristic of Bact. abortus. Also the growth on serum agar was not in such compact granules as is the case with typical Bact. lipolyticus, which made it possible to obtain a suspension suitable for the agglutination test. A suspension of Strain zy, representing this atypical group, was agglutinated in a 1 : 40 dilution of Bact. abortus antiserum.

Altogether there were recognized 5 groups of atypical Bact. lipolyticus, as shown in Table 4. All the groups agreed with the typical strains in cultural characteristics, and varied from the typical strains only slightly in biochemical reactions, the most conspicuous variation being the failure of some of the strains to decompose butter fat.

PREVALENCE OF BACT. LIPOLYTICUS IN COW'S MILK

It has been noted that Bact. lipolyticus was isolated from the milk of 6 of 24, or 25%, of the cows that had not aborted, and from 8 of 12, or 66.6%, of the cows that had aborted, as a result of natural infection, and from 2 of 4, or 50%, of the cows that had aborted after inoculation. (These figures include atypical strains of Bact. lipolyticus.) The numbers per c c were noticeably higher in the milk of cows that had aborted. Judged by these figures, Bact. lipolyticus is apparently inclined to accompany or follow infections of virulent strains of Bact. abortus. It is interesting in this connection to note that the udder of inoculated Cow 50, which is badly infected with Bact. abortus, harbors Bact. lipolyticus in about equal numbers. On the other hand, no Bact. lipolyticus have been found in the material from the udder of Cow 51, which is also badly infected with Bact. abortus.

Udder Flora of a Herd in which Exists an Epizootic of Abortion

After the data which forms the basis of this report up to this point had been collected, Herd A, which supplied all the samples of milk from cows which had not aborted, became badly infected with Bact. abortus, and an outbreak of abortions occurred, beginning about 3 months after the milk examinations reported in Table 1 were made. Nine of the 12 cows discussed in this paper which had aborted as a result of natural infection (Table 2) belonged also to the same herd, in which there were occasional abortions for several years previous to the outbreak. The milk examinations made in Sept., 1917, as reported in Table 2, were made about 2 months after the outbreak began. During those 2 months, 3 abortions had occurred. Since then several abortions have occurred every month. In the latter part of Jan., 1918, samples of milk for bacteriologic examination were obtained from 12 cows

which had aborted during the preceding 4 months, and at the same time samples of milk were obtained from 9 cows of the same herd which had not aborted. It was thought that the additional data might substantiate the conclusions already drawn. But the results of the examinations revealed so great a change in the udder flora of all the cows whose milk was examined, that it appears to be logical to discuss these results separately instead of including them in Tables 1 and 2.

The results of the bacteriologic examinations are summarized in Table 5. There was no distinction between the bacterial flora of the milk from the cows which had and from those which had not aborted. Therefore all 21 samples will be considered together. There is nothing unusual about the total number of bacteria found in these samples. The percentage of samples in which staphylococci were found agrees essentially with the percentage of a large number of milk samples (192) which were found to contain staphylococci as reported in the earlier paper.[12] The percentage of the 21 samples in which streptococci were found was abnormally high. Eleven of the 21, or 52%, contained streptococci, as compared with an average of 15% of the 192 samples of milk in which streptococci were found. The 192 samples studied in the earlier work were taken from 5 different dairies. The highest percentage of samples containing streptococci from any one dairy was 27. The history of the streptococcic flora of the milk from Herd A is as follows:

In July, 1915, streptococci were found in 5 of 22, or 23% of samples.

In April, 1917, streptococci were found in 7 of 25, or 28% of samples.

In January, 1918, streptococci were found in 11 of 21, or 52% of samples

The figures show that some time during the 9 months preceding the last examination there was a general spread of infection of the udders with streptococci, which resulted in almost doubling the percentage of cows infected, and produced a percentage of more than 3 times the general average as previously determined.

The most conspicuous change in the bacterial flora of the milk from this dairy was the general infection with a streptothrix (Nocardia). Eighteen of the 21, or 85%, of the samples contained this organism. It had spread in the dairy some time during the preceding 4 months, for in the preceding September samples of milk from 6 cows of this dairy had been plated, and no streptothrix was found. The infection of this herd with streptothrix is discussed in an accompanying paper.[12]

The data obtained does not show whether the spread of the streptococcic infection preceded or accompanied the outbreak of abortions. But they show that the streptothrix infection spread after the outbreak had started. It may be that a lowered resistance resulted from the infection with Bact. abortus which prevailed in the herd, and that this lowered resistance permitted the spread of the streptococci and the streptothrix, or the same condition may underlay all 3 abnormal infections.

[12] Jour. Infect. Dis., 1918, 22, p. 373.

TABLE 5

Bacterial Flora of the Udder During an Outbreak of Abortion. All Samples Were Plated Jan. 24, 1918

	Cow No.	Date Abortion Occurred	Agglutination Tests		Total No. of Bacteria per C c	No. of Staphylococci per C c	No. of Streptococci per C c	No. of Streptothrix per C c	No. of Bact. Lipolyticus per C c	No. of Abortus-like Bacteria per C c	No. of Acid Producing Rods per C c	No. of Other Bacteria of the Morphology of Bact. Abortus per C c
			Date	Reaction of Blood Serum								
Cows which have aborted during the outbreak	17	10/11/17	11/ 4/16 11/18/17	— +	750	100	0	420	230	0	0	0
	41	12/ 5/17	11/ 4/16 10/18/17	— +	1,100	0	400	200	100	400	0	0
	44	10/13/17	11/ 4/16 10/18/17	— +	2,200	0	600	900	0	0	100	600
	47	1/10/18	11/ 4/16 10/18/17 1/10/18	— — +	2,900	0	500	2,400	0	0	0	0
	49*	1/ 1/18	11/ 4/16 10/18/17 1/ 4/18	— — +	2,500	0	0	1,400	0	0	600	500
	50	11/ 5/17	11/ 4/16 10/18/17	+ +	1,100	200	0	0	0	500	400	0
	53	12/12/17	11/ 4/16 10/18/17 12/13/17	— — +	4,500	0	1,000	3,500	0	0	0	0
	60	1/ 1/18	11/ 4/16 10/18/17	— +	1,500	400	0	900	0	0	200	0
	100†	12/25/17	11/ 4/16 10/18/17	— slight	48,000	0	45,000	3,000	0	0	0	0
	105	11/16/17	11/ 4/16 10/18/17	— —	290	40	0	200	0	0	50	0
	106	1/13/18	10/18/17	+	1,000	500	0	700	0	0	0	0
	110	10/30/17	11/ 4/16 10/18/17	— +	3,150	750	300	1,500	0	0	0	600
Cows which have not aborted during the outbreak	14		11/ 4/16 10/18/17 1/25/18	— — —	1,350	200	150	0	1,000	0	0	0
	54		11/ 4/16 10/18/17 1/25/18	— — slight	1,150	100	200	450	400	0	0	0
	56		11/ 4/16 10/18/17 1/25/18	— — —	1,200	300	0	900	0	0	0	0
	58*		11/ 4/16 10/18/17 1/25/18	— — —	1,060	0	0	280	0	0	500	280
	59		11/ 4/16 10/18/17 1/25/18	— — slight	290	0	0	160	50	0	0	80
	61		11/ 4/16 10/18/17 1/25/18	— — —	1,130	240	90	500	150	150	0	0
	64*		11/ 4/16 1/25/18	— —	3,400	800	0	1,900	0	0	700	0
	201		11/ 4/16 10/18/17 1/25/18	— — slight	36,000	8,000	28,000	0	0	0	0	0
	205		11/ 4/16 10/18/17 1/25/18	— — —	210	0	70	140	0	0	0	0

* The sediment contained red blood corpuscles.
† The sediment was puslike.

It was previously noted that there was no difference between the bacterial flora of the milk of cows which had aborted and those which had not. Bacteria of the morphology of Bact. abortus were isolated from the milk of 66.6% of the cows which had aborted, and from an equal percentage of those who had not aborted. The percentage is lower than in the case of the cows which had aborted as reported in Table 2. But in one case, at least, that of Cow 100, the failure to find organisms related to Bact. abortus can be accounted for by the very large numbers of streptococcus colonies which grew on the plates. Two strains of Bact. lipolyticus were found in the milk of this cow in an examination made 9 months before (Table 1). The same statement applies to Cow 201. For the preceding 4½ years she had been a conspicuous "carrier" of Bact. lipolyticus. It was from her milk that the organism was first isolated, and this was the first examination since then that it failed to grow on the plates. It is most probable that the organism was still eliminated in the milk but that the very great numbers of colonies of streptococci overgrew it. In spite of the failure to isolate abortus-like organisms from Cow 201, bacteria of the morphology of Bact. abortus were isolated from 66.6% of the cows which had not aborted. The percentage is 25% higher than it was for the same herd 9 months previous, indicating that during an outbreak of abortion, the organisms of similar morphology which accompany an infection of the virulent strain spread in the udders of all the cows of the herd.

No typical Bact. abortus could be isolated from any of the 21 samples of milk, although the blood serum of all 12 cows reacted positively to the agglutination test. The failure to isolate them from the milk during an outbreak of abortion strengthens the evidence that the number of virulent Bact. abortus eliminated in cow's milk is not large.

The variation from the typical Bact. abortus was wider in some of the strains with morphology of Bact. abortus which were isolated from these 21 samples of milk than had been found in any of the strains previously studied. Particularly the property of vigorous acid production from the sugars was observed in strains from 7 of the cows. This property had not been observed before in any of the abortus-like organisms which had been studied. In dextrose, maltose, sucrose, and lactose broth cultures with an initial hydrogen-ion concentration of 6.5 P_H, the hydrogen-ion concentration was increased to

about 4.4 P_H. The growth on agar slope resembled Bact. lipolyticus, but in agar shake cultures the colonies were scattered throughout the depth of the agar. On account of these wide variations from the characteristics of Bact. abortus and Bact. lipolyticus the acid-producing rods would not have been considered in connection with those species, except for the facts that 4 of the strains showed the reduction of the hydrogen-ion concentration (about 1.0 P_H) in plain broth culture which is characteristic for Bact. abortus, and the suspensions of 2 of these strains subjected to the agglutination test with Bact. abortus antiserum showed a slight reaction (in the 1:80 or 1:40 dilution). There is no reason to believe they have any pathologic significance, but the fact that they were isolated from 7 of the 21, or 33.3%, of the samples of milk shows that the conditions which permit an infection with Bact. abortus permit also the growth in the udder of a large group of widely varying organisms of similar morphology.

SUMMARY

Bact. lipolyticus and other abortus-like bacteria were isolated from the milk of 10 of 24, or 41.7% of cows which had not aborted. The cows belonged to a herd in which there was an occasional abortion, but no general outbreak.

The same kinds of bacteria were isolated from the milk of 100% of 12 cows which had aborted as a result of natural infection. Bact. lipolyticus was cultivated from the milk of 66.6% of these cows, and other abortus-like bacteria were cultivated from the milk of 50% of them.

Typical virulent strains of Bact. abortus could not be isolated from the milk of either of the groups mentioned above.

Typical Bact. abortus was found to be present in very large numbers in the milk of 2 cows that had been repeatedly inoculated with a mixture of strains of that organism. It was found only once, in rather small numbers (450 per c c) in the milk of a cow which had aborted after being inoculated once with the same mixture of strains of Bact. abortus. It was not found in the milk of another cow which aborted after receiving one inoculation.

The data indicate that virulent strains of Bact. abortus are not eliminated continuously in large numbers in the milk of cows which have aborted, even though the blood serum continues to react positively to the agglutination test.

The characteristics of Bact. lipolyticus and other abortus-like bacteria are described, and their relation to the typical Bact. abortus is discussed. The possibility that some of these strains may cause abortions in those cases in which the blood serum reacts negatively to Bact. abortus antigen is also discussed.

The bacterial flora of the udders of a herd in which there existed an outbreak of abortions was found to be abnormal in the large number of udders which were infected with streptococci, and it was also abnormal in showing a general infection with a streptothrix. Abortus-like bacteria were found in 66.6% of the samples of milk. The abortus-like bacteria included 7 acid-producing strains which had never before been found.

A STREPTOTHRIX (NOCARDIA) INFECTION OF COWS' UDDERS

ALICE C. EVANS

From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture, Washington, D. C.

Four and one-half years ago a study of the bacterial contamination of cow's milk as it comes from the udder was undertaken by the writer. At that time a number of samples of milk from the Dairy Division herd at Beltsville, Md., were studied and 2 or 3 times each year since a number of samples (usually from 20-30) from this herd have been carefuly examined. At the time of the last examination of samples, in Jan., 1918, it was found that some time since Oct., 1917, when the preceding examination was made, there had been a general infection of the udders with a streptothrix (Nocardia). It was isolated from 18 of 21, or 85.7%, of the samples studied. The number of streptothrix colonies which grew on the plate indicated that the numbers of these organisms in the samples of milk when it was drawn varied from 140-2,600 per c c. It had never before been isolated from the milk of this dairy.

A search through the literature yielded only 2 references to the findings of streptothrix in milk. One was in the writer's paper[1] on the "Bacteria of Milk Freshly Drawn from Normal Udders." There it was reported that "A streptothrix was found in 6 of the 28 samples of milk from Dairy 3. In one sample there were 5,000 of these organisms per cubic centimeter." Thöni[2] reported that he found streptothrices in the market milk of Bern, Switzerland, and Barthel,[3] a Swedish investigator, reported finding them in milk, but he found them also in the air of the stable, and therefore concluded that those in the milk were due to air contamination. Since streptothrices are common saprophytes, it is probable that those isolated by Thöni got into the milk after it was drawn. Presumably, then, the streptothrix infection of the udder was unusual, for otherwise it would have been noted by others of the investigators who have studied the bacterial flora of aseptically drawn milk.

Received for publication May 11, 1918.

[1] Jour. Infect. Dis., 1916, 18, p. 437.
[2] Gesundheitsamt, 1914, 5, p. 9.
[3] Milch-Zeitung, 1903, 32, p. 658.

Description of the Organism

Morphology.—In a young vigorously growing culture, the streptothrix consists of long branching mycelial threads, which afterward break up into rod forms of various lengths. The rod forms are in turn converted into coccoid forms which occur isolated or in chains held together by the remnants of the old mycelium. The coccoid forms are the result of "chain sporulation." If the medium is dry, a powdery growth appears, in which true conidia are formed at the ends of lateral hyphae, together with all the other forms the organism may assume. The morphology of this milk streptothrix is splendidly illustrated in the colored plates of Claypole's "Classification of the Streptothrices,"[4] to which the reader is referred. All the forms represented in Figures 1, 2, 3, and 4 of Plates 8 and 9 were found also in cultures of the milk streptothrix.

Straining.—The organism is gram-positive. The coccoid bodies and conidia are acid-fast when stained by carbolfuchsin and decolorized with 2% hydrochloric acid in 95% alcohol, as recommended by Jordan[5] for tubercle bacilli. They are also acid-fast when decolorized with 5% nitric acid, as recommended by Hiss and Zinsser.[6] But they are not acid-fast when decolorized with 25% nitric acid, according to the method used by Claypole.[4]

Cultural Characteristics.—On agar slope the growth is usually in discrete colonies about 1½ mm. in diameter. The colonies are wrinkled, with the ridges radiating from a depressed center. Often there is a triangular opening in the center of the colony. Sometimes the growth is confluent, with a wrinkled surface. It is tough and adheres to the agar. The growth itself is of a cream buff color (Plate 19″ YO-Yd of Ridgeway's Color Standards and Nomenclature), and the agar is slightly browned in old cultures. In agar shake culture growth takes place on the surface and on the wall of the tube for about 10 mm. above the surface. On the upper part of slope cultures, where the agar is thin and dry there is often a white powdery growth. It is in this efflorescent growth that the true conidia may be found at the ends of aerial hyphae. In broth cultures the growth is in the form of granules which do not cloud the medium. On potato there is an abundant brownish growth with a honeycombed surface.

Biochemical Reactions.—The streptothrix was quite inert toward the medium in which it was cultivated. It did not attack dextrose, maltose, saccharose, lactose, starch or mannite in broth culture, nor did it reduce the hydrogen-ion concentration of the medium. It failed to decompose nitrate and asparagin, and failed to grow on gelatin at 20 C. It rendered litmus milk alkaline with a slow action.

Thermal Death Point.—The thermal death point of the organism was determined in a whole milk culture 8 days old. It was killed by a temperature of 56 C. for 30 minutes. A smear of the culture for microscopic examination was stained according to the Gram-Weigert method to determine what forms of the streptothrix were present. The long-branched mycelial threads predominated, with a few rods and coccoid forms. No conidia were found and it is probable that there were none in the culture. But it can be assumed that the 8-day culture contained forms as resistant to heat as would occur in milk at the time of pasteurization; therefore, the heat of ordinary pasteurization can be regarded as sufficient to kill the organism.

[4] Jour. Exper. Med., 1913, 17, p. 99.
[5] General Bacteriology, 1915, p. 48.
[6] A Text Book of Bacteriology, 1915, p. 105.

DISCUSSION

Streptothrices are common saprophytic and parasitic organisms which occur in soil and water, and are associated particularly with herbage. They are the cause of many diseases and infection has in many cases been traced to herbage. Cattle are the most susceptible animals, and the most common diseases caused are lumpy jaw and pneumonia. Foulerton[7] studied 53 cases of streptotrichosis in human beings and found the most common infections were about the mouth or in the neck, as the result of oral infection. The next common seat of apparent primary infection was the appendix, and next was pulmonary infection. In the tropics Madura foot is a common disease caused by a streptothrix. Claypole[8] states that "Streptotrichosis is more common than generally supposed and frequently not differentiated from other infections."

Streptothrices belong to the higher bacteria which form connecting links between the fission fungi and the true fungi, with B. leprae and B. tuberculosis as the most closely related forms of fission fungi. Claypole[4] recognized 11 species of streptothrix pathogenic to human beings. The 11 species showed a series of gradations between Streptothrix hominis III at one end and B. leprae and B. tuberculosis at the other end of the series. The milk streptothrix agreed in morphology, cultural characteristics, biochemical reactions and staining properties with Streptothrix hominis III, which was first described by Foulerton.[7] The milk streptothrix therefore agreed with the species nearest related to the true fungi with long, branched mycelial threads, and common formation of conidia. It was one of the least acid-fast organisms of Claypole's group, and serologically was the most remotely related to B. tuberculosis.

Foulerton[7] isolated Streptothrix hominis III from 3 cases of appendicitis. Any comment on the possible sanitary significance of large numbers of a similar organism in milk would be mere conjecture. Animal inoculation would not give reliable information on this point, for numerous investigators who have studied the streptothrices agree that pathogenicity is not a reliable specific character. There is reason to believe that the organism was not living as an entirely harmless parasite in the cows' udders. Three of the 18 samples of milk from which the streptothrix was isolated gave a sediment containing red

[7] Lancet, 1910, 178, p. 626.
[8] Arch. Inter. Med., 1914, 14, p. 104.

blood corpuscles. A fourth sample from which the streptothrix was isolated gave a puslike sediment, but it also contained streptococci in numbers great enough to account for the abnormal condition. Apparently, however, the streptothrix was not causing any serious udder trouble, for a month after the samples of milk were examined the dairyman in charge reported that he had not noticed any unusual condition.

The finding of streptothrices in milk does not add to the precautions which should be observed to safeguard against infection by food substances. It merely adds a fourth organism to the 3 types of bacteria of a suspicious character already known to occur in milk freshly drawn from normal udders — the streptococci, the staphylococci, and the rodforms of the Bact. abortus type. Strains of all 3 of these organisms, which produced disease when inoculated into experimental animals, have been found in milk. Melvin's[9] statement in regard to Bact. abortus can be applied also to the streptothrix: "Although we do not know what effect it may have on human beings, we cannot afford to assume that they are not affected by it." The streptothrix may prove to be still another source of danger in the use of raw milk as food, and may furnish an additional reason for taking advantage of the safeguard afforded by pasteurization.

[9] Twenty-Eighth Ann. Report Bur. An. Ind., Department of Agriculture, 1911, p. 137.

THE EMPLOYMENT OF URIC ACID SYNTHETIC MEDIUM FOR THE DIFFERENTIATION OF B. COLI AND B. AEROGENES

Stewart A. Koser

From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University

In the course of an investigation on the utilization of simple nitrogenous compounds of definite chemical composition, it was observed that B. aerogenes was able to multiply and grow luxuriantly in a medium which contained uric acid as the only source of nitrogen. B. coli, on the other hand, failed to develop. Because of the recent interest attached to these 2 types this point was investigated further, and a number of different strains of each organism was subjected to this test.

Several chemically definite mediums were employed. The most satisfactory results were obtained with the following:

Distilled ammonia-free water	1,000 c c.
NaCl	5.0 gm.
$MgSO_4$	0.2 gm.
$CaCl_2$	0.1 gm.
K_2HPO_4	1.0 gm.
Glycerol	30.1 gm.
Uric acid	0.5 gm.

This combination gives a colorless and clear medium. It was filled into ordinary test tubes and sterilized in the autoclave at 13-15 lbs. extra pressure for 15 minutes. A slight turbidity was apparent after autoclaving, due presumably to a finely divided precipitate of calcium sulphate. On cooling, the solution became clear.

In this medium B. aerogenes grew luxuriantly and soon produced a dense clouding. B. coli failed to develop and the tubes remained clear. In all, 124 different cultures of both B. coli and B. aerogenes were obtained and their ability to grow in the above medium tested. For these cultures the writer is indebted to Mr. C. C. Chen of this laboratory. The cultures of the colon bacillus were of fecal origin, while those of B. aerogenes had been isolated from various soils. Only those strains which were typical of either type were employed. At the same time these strains were also inoculated into the peptone-dextrose-

Received for publication May 15, 1918.

dipotassium phosphate medium of Clark and Lubs.[1] The results of the methyl red and Voges-Proskauer tests in this medium were compared with the ability to develop in the uric acid solution. A striking correlation was found, which is summarized in the accompanying table.

TABLE 1

INCUBATED 4 DAYS AT 37 C.

Cultures Employed		Uric Acid Medium		Methyl Red Test		Voges-Proskauer Reaction	
		Growth	No Growth	Positive	Negative	Positive	Negative
B. coli	74	0	74	72	2	0	74
B. aerogenes	50	50	0	0	50	50	0

Within the first 24 hours the cultures of B. aerogenes attained a fair degree of turbidity and could easily be distinguished from the clear tubes which had been inoculated with the colon bacillus. As the cultures grew older the increased luxuriance of B. aerogenes accentuated this difference and the contrast was more striking.

The basic potassium phosphate is necessary to dissolve the uric acid, since this substance is insoluble in water. The glycerol also seems to aid in its solution. After the addition of the uric acid, the P_H value, as determined by the colorimetric method, was found to be 6.7-6.8. This slight acidity prevents the magnesium and calcium from precipitating as phosphates. In an effort to simplify the medium the magnesium sulphate and calcium chlorid were omitted. As the B. aerogenes cultures grew less luxuriantly in the absence of these salts this modification was discarded.

On the addition of 1.5% of washed shred agar to the solution mentioned in the foregoing an agar medium was obtained on which the same distinction between the 2 types may be brought out. Hypoxanthin hydrochlorid, when substituted for the uric acid, gave the same differentiation, although B. aerogenes appeared to grow less luxuriantly than in the uric acid medium. As the amount of available hypoxanthin was limited, a few tests only were performed.

Certain precautions were taken to exclude extraneous sources of nitrogen from the synthetic medium. All tubes were inoculated very lightly from 24-hour agar cultures. In addition, an effort was made to prevent, as far as possible, the absorption of gaseous ammonia by the medium, since B. coli, as well as B. aerogenes, is capable of grow-

[1] Jour. Inect. Dis., 1915, 17, p. 160.

ing in the presence of an ammonium salt, such as ammonium phosphate.

The distinction between these 2 types would seem to be due to the fact that B. aerogenes is capable of attacking the purin ring and utilizing the nitrogen thereof, while B. coli lacks this power and, since there is no source of available nitrogen, fails to develop. This differentiation is of interest in that it is one of fundamental nitrogen nutrition and not of carbohydrate metabolism, as are those distinctions based on the CO_2 : H ratio, the attainment of a certain hydrogen-ion concentration, or the production of acetyl methyl carbinol.

DIFFERENTIATION OF TYPHOID AND PARATYPHOID A AND B BACILLI BY A DEXTRIN-INOSITE MEDIUM

Florence Hulton-Frankel

From the Harriman Laboratory, Roosevelt Hospital, New York.

Numerous cases of paratyphoid in the hospital made it necessary to use, beside the Russel double sugar agar, a second medium for differentiating between the typhoid bacillus and paratyphoid bacilli A and B. Since paratyphoid bacillus B ferments inosite with gas formation and paratyphoid bacillus A does not, the inosite medium of Weiss and Rice[1] was used. This made a second planting necessary, and it seemed a good plan, if possible, to get further confirmatory evidence as to whether the typhoid bacillus ferments dextrin and the paratyphoids do not, so a medium containing both carbohydrates was used.

The medium was an ordinary 3% infusion agar, with the usual typhoid reaction of about + 0.7 acid. The agar was sterilized in the autoclave and if there was any change in reaction it was readjusted to + 0.7 acid; 1% of both dextrin and inosite were added (other proportions were tried but did not give satisfactory results) and a sterile litmus solution (Grubler's) was added till a light violet color was obtained, and the medium tubed. The medium was given one sterilization (Arnold), as further heating tends to break up the carbohydrates, and slanted. The slants were incubated for 24 hours and any tubes discarded which showed growth. This should be the usual procedure for testing all mediums but is especially necessary in this case where such short sterilization was given.

The dextrin used was Merck's C.P. and the inosite was made in this laboratory according to Nelson.[2] Dextrin represents a heterogeneous group of gums varying in complexity from amylodextrin approaching starch in its reactions to achrodextrin. The dextrins which seem to be acted on by the typhoid bacillus must be erythrodextrin as every sample containing the lower dextrins gave results while the amylodextrin did not. The Merck preparation is a mixture of all of them. Erythrodextrin can be easily prepared by allowing saliva to act on soluble starch until the digestion mixture no longer gives a starch reaction. The dextrin is then thrown down by alcohol, dried and procured as a white powder.

The stools and urine were plated on brilliant green medium of Teague[3] or Endo plates and suspicious colonies fished and planted on Russel double sugar agar and dextrin inosite medium. With the dextrin-inosite medium the typhoid bacillus gave a violet slant with a red butt in about 12 hours and an extremely decolorized butt in 24 hours, while the paratyphoid bacillus B gave the same reaction with the additional formation of gas in the butt and the paratyphoid bacillus A did not react at all, merely giving a good growth on the slant.

Received for publication May 6, 1918.

[1] Jour. Med. Research, 1917, 30, p. 403.

[2] Jour. Am. Chem. Soc., 1915, 37, p. 1552.

[3] Jour. Infect. Dis., 1916, 18, p. 1. Ibid., 1917, 21, p. 145.

The typhoid bacilli that were tried on the medium had mostly been cultivated on artificial mediums for some time and seemed to have lost some of their powers of fermentation, but after cultivation through broth they regained them. This condition did not arise in those cases in which the organisms were freshly isolated from urine or stools. Of the strains tried, all 23 eventually produced a good growth on the slant and acid in the butt of the tube, with the final reduction of the litmus.

The 9 strains of the paratyphoid bacillus B that were used in this investigation all gave a violet slant with well marked growth and a decolorized butt with well marked gas formation.

All 8 of the strains of paratyphoid A used gave a good growth on the dextrin-inosite medium with neither gas nor acid formation.

Other members of the typhoid-colon-dysentery group were tried on this medium with the following results: B. dysenteriae, Shiga-Kruse type and Hiss-Russel type, gave no reaction, while Flexner and Rosen types gave acid formation.

B. coli communior gives acid and gas formation while B. coli gives no reaction. B. aerogenes gives acid and gas formation. B. murium and B. pullorum give no reaction, though Weiss and Rice report one group of B. typhi-murium as negative on inosite and another as positive.

All the strains tried were identified by the Gram stain and agglutination with specific serum in dilutions up to 1 : 1,000 both before and after planting .

The dextrin-inosite medium differentiates between the typhoid bacillus and paratyphoid bacilli A and B, although it does not differentiate between the paratyphoids and the colon bacilli. In conjunction with Russel double sugar agar it gives confirmatory evidence of the identity of the typhoid bacillus and distinguishes between the paratyphoid bacilli A and B.

SUMMARY

The typhoid bacillus ferments dextrin-inosite with acid formation.

The paratyphoid bacillus B ferments dextrin-inosite medium with acid and gas formation.

The paratyphoid bacillus A does not ferment dextrin-inosite.

The dysentery group falls into two groups; the Shiga-Kruse and Hiss-Russel types do not ferment dextrin-inosite, while the Flexner-Rosen types do.

B. typhi murium and B. pullorum do not ferment dextrin-inosite.

B. aerogenes ferments dextrin-inosite with gas formation.

THE ACTION OF CERTAIN ANTISEPTICS, TOXIC SALTS, AND ALKALOIDS ON THE BACTERIA AND PROTOZOA OF THE INTESTINE OF THE RABBIT

A. K. Balls and José D. Moral

From the Department of Bacteriology, Columbia University, New York

In the summer of 1917, our work with some newly proposed antiseptics and recent literature regarding their properties suggested that useful conclusions might be drawn by comparing the antiseptic action of these and other typical bactericides on both bacteria and protozoa, those of the intestine of the rabbit being used for reasons stated subsequently. The work was amplified by the investigation of certain typical antiseptics, both organic and inorganic, and also of various alkaloids in current use against diseases produced by protozoa.

Historical

Ehrlich[1] has proposed as a fundamental principle of chemotherapy that the protoplasm of the parasite is equipped with specific chemoreceptors capable of combining with certain molecular configurations while the protoplasm of the host possesses fewer if any such specific receptors, and is accordingly less influenced. If the substance combining with the receptors in question be poisonous to the protoplasm, then the toxic effect will be much more evident on the parasite than on the host. This hypothesis has been supported by the observed specific action of salvarsan against treponema and spironema, and of atoxyl against trypanosoma. The idea explains the action of the salts of quinin on the plasmodium of malaria and the action of potassium antimonyl tartrate on leishmania. These effects are based on the comparative resistance of protozoa and tissue, and they were worked out some time ago. The corresponding problem of the resistance of bacteria and tissue has only recently received attention in the experiments of Carrel, Dakin, Daufresne, Dehelly and Dumas.[2]

In view of the conception of Ehrlich regarding chemoreceptors, it seems logical to regard those of human tissue and of treponema as differing considerably. The difference in the toxicity of salvarsan toward human tissue and treponema can thus be explained. The difference in the receptors of two closely related organisms, however, cannot be as great, and a substance toxic to one organism may exhibit approximately the same toxicity to others nearl/ allied to it.

Between bacteria and protozoa one would hardly expect extreme differences in behavior toward a toxic agent. For the demonstration of different susceptibility on the part of protozoa and bacteria to a substance injuring them, it is necessary to carefully consider such factors as the concentration and the time of action of the material tested. Pathogenic bacteria as a class are probably closely related, and while the effect of certain substances, principally dye-stuffs,

Received for publication May 18, 1918.

[1] Die Experimentelle Therapie der Spirillosen, 1910.

[2] Presse méd., 1915, 23, p. 397.

has been found to vary on different varieties of bacteria, these variations are not so much in the bactericidal power of the substance employed as in the finer detail of its effect on the rate of growth, causing the inhibition of some bacterial species, while favoring the growth of others as seen in differential mediums.

In 1902, Conradi and Drigalski[3] directed the use in their medium for the isolation of the typhoid bacillus of 0.001% of crystal violet, to inhibit the growth of many other bacteria. Loeffler[4] used malachite green in a medium for the same purpose. This dye inhibits the growth of the colon bacillus while exerting no such action on the typhoid bacillus. Uhlenhuth and Xylander[5] working in another field found that antiformin—a solution of NaOCl containing some Na_2CO_3 and differing essentially in this latter constituent from Dakin's solution—while toxic to almost all bacteria exerts little effect on the tubercle bacillus, and therefore forms the basis of a method for isolating this organism in pure culture. Churchman and Michael[6] found that an addition of gentian violet in a dilution of 1 : 100,000 inhibited the growth of many bacteria, while allowing others to grow luxuriantly. These authors proposed a classification of bacteria into two groups, violet positive and violet negative on a basis of this reaction. Brown and Gilmour[7] studied the relationship between chemical constitution and bactericidal action, particularly with a view to the possible use of bactericidal dye-stuffs in clinical practice. They found that serum diminished greatly the action of such powerful bactericidal dyes as brilliant green, an effect similar to the action on mercuric chlorid under the same conditions although probably not from the same cause. Brown, Gilmour and Mackie[8] present a new method of isolating the typhoid bacillus by means of brilliant green in fluid medium. With a proper dilution of the dye only the typhoid organism will survive. R A. Lambert[9] showed that tissue cells and bacteria were killed with equal ease by potassium cyanid, tricresol, hydrogen dioxid and alcohol, but that with iodin the cells were more resistant. Inman[10] in the same year stated that the measurement of the antiphagocytic action of antiseptics was an expression of their general toxicity to living matter, and Leitch[11] uses brilliant green as a stimulant of the formation of granulation tissue. Browning, Gulbraneen, Kennaway and Thornton[12] claim that brilliant green and flavine are substances having little effect on the processes of phagocytosis, and are all the more valuable because interfering little with normal methods of combating infection; they regard this property as extremely important in determining the utility of an antiseptic to be used on infected tissue.

Generally speaking, it may be assumed that protozoa are of a more complex structure than bacteria and it might be possible to discover differences in the reaction of bacteria and of protozoa to the same antiseptic substance. Should such a condition be found, three interesting possibilities would result: (1) the possibility of separating protozoa in a new way from a culture contaminated by bacteria; (2) the possi-

[3] Ztschr. f. Hyg. u. Infektionskrankh., 1902, 39, p. 283.
[4] Deutsch. med. Wchnschr., 1906, 32, p. 289.
[5] Berl. klin. Wchnschr., 1908, 45, p. 1346.
[6] Jour. Exper. Med., 1912, 16, p. 822.
[7] Jour. Path. and Bacteriol., 1914, 18, p. 144.
[8] Ibid., p. 147.
[9] Jour. Exper. Med., 1916, 7, p. 683.
[10] Jour. Royal Army Med. Corps, 1916, 27, p. 500.
[11] Brit. Med. Jour., 1916, 1, p. 236.
[12] Ibid., 1917, 1, p. 73.

bility of removing from vaccine virus the bacteria which frequently contaminate it, and (3) the possibility of developing a method for studying the therapeutic action of the numerous preparations recommended for the treatment of human protozoan intestinal infections.

During the course of our work on the purification of vaccine virus the work of Krumwiede and Watson[13] appeared. These investigators have solved the problem of vaccine purification by the selective action of brilliant green in a convenient concentration against the contaminating bacteria without diminishing the viability of the virus. We think it possible that future studies with flavine may lead to similar results.

We confine ourselves in this paper to the susceptibility of bacteria and protozoa to the same toxic agents, trusting it may be possible to elaborate many of the details later.

The rabbit was selected for investigation on account of the abundant and practically constant presence of Giardia cuniculi in the intestine, generally without association with other protozoa and because of the similarity of this parasite to other species of Giardia inhabiting the intestines of rats, which have frequently been found as etiologic factors in the intestinal disorders among soldiers in trench warfare.[14]

METHODS

Chosen dilutions of the antiseptic in sterile salt solution were placed in small tubes arranged in series of 10 including a control; 0.1 c c of the contents of each tube was mixed with an equal volume of liquid material containing living protozoa and bacteria, so that the final dilution of the antiseptic was half that of the first series of tubes. Precautions were taken to insure sterile environment for the mixture. As a control, sterile salt solution was used.

Prior to mixing the dilutions of the antiseptic with the protozoa and bacteria, a series of hollow ground slides was prepared, so that as soon as the mixture had been made, a portion could be placed in a hanging drop and examined under a microscope. By making these operations at regular intervals of 1 minute, the whole series of 10 tubes could be started in 10 minutes, and by making observations 1 minute apart thereafter, the various preparations could be inspected each at 10, 20, and 30 minutes after mixing, and so on until the end of the experiment.

The microscopic preparations were studied by a strong dry lens with regard to the motility of the lamblia. When no diminution of the motility was observed, the results are recorded as +; when diminution of motility was seen, the result is recorded as ±; and when no movement could be observed either of the body or the flagella, the result is recorded as 0. At this point we assume the protozoa to be dead.

During the course of the experiments and particularly toward the close when the results are of most moment, loopfuls of the mixture of protozoa and bacteria were inoculated into sterile 1.5% acid extract broth at different times.

[13] Jour. Infect. Dis., 1918, 22, p. 118.
[14] Fantham, H. B., and Porter, Annie: Brit. Med. Jour., 1916, 2, p. 139.

These tubes after an incubation of 48 hours showed by growth or sterility whether or not the bacteria had been also killed at the particular time the subinoculation was made.

The mixture of protozoa and bacteria was obtained as follows: A rabbit was killed and the abdomen opened rapidly. From various parts of the small intestine, a few drops of the liquid contents were removed, and observed in a hanging drop to ascertain in which portion of the intestine the greatest number of lamblia were to be found. This part was removed from the animal and the contents collected, mixed with an equal amount of salt solution, and filtered through cotton. The filtrate contained many motile lamblia, and of course many intestinal bacteria also.

INFLUENCE OF TEMPERATURE

The observations recorded were made at room temperature, varying but slightly from 20 C. At 37.5 the action of the antiseptic is in general more rapid. Comparisons with brilliant green show that a dilution of this dye which did not kill the protozoa in 1 hour at room temperature did kill in 15-20 minutes at incubator temperature. This fact must be taken into account when it is desired to consider the action of an antiseptic in the animal body. After determining the resistance of the organisms at room temperature, a second experiment with dilutions somewhat weaker than the first must be made at 37.5 C.

OCCURRENCE OF GIARDIA CUNICULI IN THE INTESTINE OF RABBITS

In the animals used in this laboratory and which are obtained for the most part from New York, New Jersey and Pennsylvania, this protozoon is a nearly constant inhabitant of the large intestine, but is not always found in the small intestine. The occurrence seems to vary somewhat with the season, being less in winter. Large amounts of good material for this work were found in about 80% of the animals used during the summer and autumn. During the winter only about 15% contained sufficient lamblia in the small intestine to be useful.

Frequently during immunization for the preparation of agglutinating serums, especially when using bacteria of the colon-typhoid group as antigens, the animals present enteric symptoms with fluidified feces. In these cases we always have found an enormous multiplication of the protozoa in the intestine, even in winter, and this material was found specially adapted for our work. The number of protozoa in a single field of the hanging drop preparations naturally varies as outlined. Frequently such fields contained 20 or 30 organisms, and as a criterion of the applicability of the material for our work, we rejected that which when set up in a control as described did not show at least 5 actively motile lamblia during 1 minute's observation.

RESULTS

In the tables the results of this method are presented for 23 substances. The selective action on bacteria as opposed to protozoa we have found to be true of 2 proprietary antiseptics of the chlorin series (chloramin-T and "halazone"),* with one of dyes, brilliant green, and with copper in the cupric state.

* Chloramin-T, chlorazene, and halazone are derivatives of substituted benzene-sulphonamids containing chlorin. This chlorin in many respects acts similarly to that in Dakin's solution and is the "active ingredient" of the antiseptics. That the action is modified from that of a simple hypochlorite, however, is evident from the fact that Dakin's solution (Table 1, 14) exhibits no similar selective action for bacteria.

TABLE 1

The Action of Various Antiseptic Substances in Different Dilutions on Both Protozoa and Bacteria

Antiseptic	Dilution	Time of Exposure: 10′		20′		30′		40′		50′		60′		70′		80′		90′		Control
		P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	
Picric acid	1:400	0	+	0	+	0	0													Alive for 160′
	1:500	0	+	0	+	0	+													
	1:700	±	+	0																
	1:800	±	+	±	+	±	+	0	+											
	1:900	+	+	+	+	±	+	±	0	0	0									
Tricresol	1:700	0		0		0														
	1:800	±	+	0	+	0	+	0	0											
	1:900	+	+	+	+	±	+	±	+	0	0									
	1:1,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	
	1:1.100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Argyrol	1:100	0		0																
	1:200	0		0																
	1:400	0		0																
	1:600	+	+	±	+	±	+	0												
	1:1,000	+	+	+	+	±	+	0												
	1:2,000	+	+	+	+	+	+	±	+	0										
Chloramin-T	1:1,400	0		0		0														Exper. a: control + for 130′. Exper. b: control ± in 85′
(Exper. b)	1:1,500	0		0		0														
	1:1.600	+	0	+	0	±	0	±	0	±	0	±	0	±	0	0				
	1:1,700	+	+	+	0	±	0	±	0	±	0	±	0	±	0	±	0			
	1:1,800	+	+	+	0	+	0	+	0	+	0	+	0	+	0	+	0	+		
Chloramin-T	1:2,000	+		±	+	±	+	±	0	±	0	0								
(Exper. a)	1:2,600	+		±		+		±	+	±	+	±	0	0						
	1:3,000	+	+	+	+	+	+	+	+	±	+	±	+	0	+					
	1:4,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Brilliant green	1:200	0		0		0														
(Exper. a)	1:500	0		0		0														
	1:1,000	±	0	±	0	0														
	1:2,000	+	+	+	0	+	0	±	0	±	0	±	0	±	0	±	0	±	0	
	1:4,000	+	+	+	+	+	+	+	+	+	0	+	0	+	0	±	0	±	0	
Brilliant green	1:1,000	0		0	0	0	0													
(Exper. b)	1:2.000	+		+	0	+	0	+	0	+	0	+								
	1:3,000	+		+	0	+	0	+	0	+	0	+								
	1:4,000	+	+	+	+	+	+	+	+	+	0	+	0							
Copper sulphate	1:200	0	0	0	0															
(Exper. a)	1:400	0	0	0	0															
	1:1,000	0	0	0	0															
	1:2,000	+	+	+	0	+	0	+	0	+	0	+	0	+	0	+	0	±		
Copper sulphate	1:2,000	+	+	+	0	+	0	+												
(Exper. b)	1:3,000	+	+	+	+	+	0	+												
Halazone	1:6,000	+	0	+	0	+	0	+	0	+	0	+	0	+	0	+	0	±	0	Alive for 120′ (+)
	1:8,000	+	0	+	0	+	0	+	0	+	0	+	0	+	0	+	0	±	0	
	1:10,000	+	+	+	0	+	0	+	0	+	0	+	0	+	0	+	0	+	0	
	1:12,000	+		+	0	+	0	+	0	+	0	+	0	+	0	+	0	+	0	
	1:14,000	+		+		+	0	+	0	+	0	+	0	+	0	+	0	+	0	
	1:16,000	+		+		+	+	+	+	+	+	+	+	+	0	+		+		
	1:18,000	+		+		+		+		+		+		+	+	+	+	+	+	
Glycerol	1:4	+		±		±	+	0	+	0	+	0	+	0	0					
	1:6	+		+	+	+	+	±	+	±	+	±	+	0	+					
	1:16	+		+	+	+	+	+	+	+	+	+	+	+	+	+				
Mercuric chlorid	1:1.000	0	0	0	0	0														
	1:1,500	0	0	0	0	0														
	1:2,000	0	0	0	0	0														
	1:2,500	0	0	0	0	0														
	1:3,000	0	+	0	0	0														
Borax	1:100	+		+		±	0	±	0	±	0	0								
	1:200	+		+		+	+	+	+	±	0	±		±		0				
	1:400	+		+	+	+	+	+	+	+	+	±	+	±	+	±	+	±	+	
	1:600	+		+		+		+		+		+		+		±		±		

TABLE 1—Continued

The Action of Various Antiseptic Substances in Different Dilutions on Both Protozoa and Bacteria

Antiseptic	Dilution	Time of Exposure																		Control
		10′		20′		30′		40′		50′		60′		70′		80′		90′		
		P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	
Gram's iodin solution	1:1,000	0		0	0	0														± in 80′
1:300	1:2,000	0		0	0	0														
	1:3,000	+	+	+	0	±	0	±		0										
	1:6,000	+	+	+	+	+	0	+	0	±		±		±		0				
Barium chlorid..........	1:200	0	0	0	0	0														
	1:400	0	+	0	0	0														
	1:1,000	0	+	0	+	0	0													
	1:2,000	+	+	+	+	±	+	±	0	0										
	1:4,000	+	+	+	+	+	+	±	+	±	+	0	0							
Carbon bisulphid........ (saturated sol.)	1:2	+	+	+	+	+	+	+	+	±	0	0								
	1:5	+	+	+	+	+	+	+	+	+	0	±	0	±						
	1:20	+	+	+	+	+	+	+	+	+	+	+	+	±						
	1:50	+	+	+	+	+	+	+	+	+	+	+	+	±						
Dakin's solution.........	1:4	±		0	0	0														
	1:8	+		+	+	+	+	+	+	±	0	±	0							
	1:16	+		+		+		+		+		+	+							
	1:32	+		+		+		+		+		+	+							
Potassium antimonyl tartrate	1:500	+		±		0	+	0	+											+ in 120′
	1:1,000	+		+		±		0												
	1:1,500	+		+		+		+		±		±		±						
	1:2,000	+		+		+		+		+		+		+		+		+		
Thymol.................. (saturated sol.)	1:2	0		0	0	0														
	1:4	±		0	+	0														
	1:6	+		+		+	+	+	+	+	+	±		±						
	1:8	+		+		+		+		+		+	+	+						
Chromic acid............	1:400	0	0	0	0															
	1:800	0	0	0	0															
	1:1,200	±		0	+	0														
Phenol..................	1:200	0	0	0	0	0														
	1:220	0	0	0	+	0														
	1:240	±	+	0	+	0														
Magnesium chlorid......	1:500	+		±		±	+	0												Alive for 150′ (+)
	1:1,000	+		+		±	+	±	0	0										
Ferric chlorid............	1:800	±		0	+	0														
	1:1,000	±		±		±	+	0												
	1:1,500	+		+		+		±												
Ferrous sulphate........	1:600	±		0	+	0														
	1:800	±		0	+	0														
	1:1,000	+		±	+	0														
Ethyl alcohol 95%.......	1:4	0		0		0														
	1:8	0		0		0														
	1:16	±		±		0	+													
	1:32	+		+		+		+		+	+	+	+	+	+	+	+	+	+	
Quinin sulphate..........	1:1,000	0		0	+	0														
	1:1,500	±		0		0														
	1:2,000	+		±		0														
	1:2,500	+		+		±		0												

Explanation: Column P: + = normal motility; ± = partial motility; 0 = absence of motility of the protozoa. Column B: + = the presence, or 0 = the absence of any living bacteria as shown by cultural tests.

TABLE 2

THE ACTION OF HALAZONE, CHLORAMIN-T AND BRILLIANT GREEN ON FREE LIVING BACTERIA AND A FLAGELLATE, COLPIDIUM SP.

Antiseptic	Dilution	Time of Exposure 10′		20′		30′		40′		50′		60′		70′		Control	
		P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B
Halazone........	1:5,000	0	0	0	0											+	+
	1:10,000	0	0	0	0											+	+
	1:20,000	±		±	+	0	+									+	+
	1:40,000	+		+		+		+		+		+	+	+	+	+	+
Chloramin-T....	1:400	0		0	0											+	+
	1:600	±	+	+	+											+	+
	1:800	±	+	0	0											+	+
	1:1,000	±		0	+											+	−
	1:1,200	±		0	+											+	−
	1:1,500	+		+		+		+	+	+	+	+	+	+	+	+	−
Brilliant green...	1:3,000	0		0		0	+									+	−
	1:4,000	0		0		0	+									+	−
	1:5,000	0		0		0	+									+	−
	1:6,000	+		+		+		+		+	+	+	+	+	+	+	−

Bacteria-protozoa mixture obtained from hay infusion. The demonstration that the protozoa were living was done by both direct microscopic examination and subsequent growth in hay infusion medium.

TABLE 3

THE ACTION OF A SINGLE ADDITION OF CHLORAMIN-T TO MATERIAL CONTAINING FREE LIVING BACTERIA AND PROTOZOA (AMEBA LIMAX SP.), AND SUBSEQUENT INOCULATION OF THE MIXTURES INTO SEMI-SOLID CABBAGE-AGAR MEDIUM IN STERILE PETRI DISHES

Antiseptic Dilution	Time of Exposure	Results After a Week Incubation at Room Temperature	
1:200	1 hour	Living bacteria but no protozoa	Only one species of sporulated bacteria
1:300	1 hour	Living bacteria but no protozoa	
1:400	1 hour	Living bacteria and protozoa	
1:500	2 hours	Living bacteria and protozoa	Several species of bacteria
1:600	2 hours	Living bacteria and protozoa	
1:700	3 hours	Living bacteria and protozoa	
1:800	3 hours	Living bacteria and protozoa	

Bacteria-protozoa mixture obtained from infusion of cabbage.

These substances are not characterized by any chemical similarity, and there is no basis for ascribing to them a common mode of action. They each probably represent a group of substances of similar action, but the variations in toxicity with slight changes in the chemical configuration we have not endeavored to work out in this paper.

Studying the action of these different substances in different concentrations against the mixture of protozoa and bacteria containing much organic matter from the intestine, we have been able to see in the case of each of these substances certain limits of dilution beyond which they are practically without action against protozoa, nevertheless the action against the bacteria is considerable. Chloramin-T in a

dilution of 1:600 kills the bacteria in 10 minutes, while the lamblia remain alive for 70 minutes in contact with the same solution. In a dilution of 1:800 the bacteria are killed in 20 minutes without any harmful effect on the protozoa observable by comparing their motility with that of the control. Halazone in 1:6,000 kills the bacteria in 10 minutes, while in 90 minutes of contact the protozoa possess only a slightly diminished motility. A solution of 1:14,000 appears to be the highest dilution in which the halazone affects the bacteria. This is much more concentrated than the ordinarily accepted highest toxic dilution, as stated by the manufacturers, a result probably to be

TABLE 4

THE ACTION OF TWO SEPARATE ADDITIONS OF CHLORAMIN-T TO MATERIAL CONTAINING FREE LIVING BACTERIA AND PROTOZOA (AMEBA LIMAX SP.), AND SUBSEQUENT INOCULATION OF THE MIXTURE INTO SEMI-SOLID CABBAGE-AGAR MEDIUM IN STERILE PETRI DISHES

Dilution After the First Addition	Exposure After the First Addition	Dilution After Second Addition (Inc. Amt. Added Previously)	Exposure After Both Additions	Results* After Second Exposure	
				P	B
(1) 1:200	90 minutes	Same. No additional Chloramin-T used	30 minutes	0	0
1:300				0	0
1:400				+	+
(2) 1:500	90 minutes	1:250	30 minutes	0	0
1:600		1:300		0	0
1:700		1:350		0	+
1:800		1:400		+	+

Bacteria-protozoa mixture obtained from infusion of cabbage.

The results of (1) and (2) by their similarity seem to indicate that failure of the chlorin antiseptic to kill bacteria is not due to its decomposition by protein, etc., bodies present, for if this were so (2) would contain more of the active antiseptic than (1) and therefore be more toxic.

* For explanation of symbols see Table 1.

explained by the presence of the partly digested protein and other organic matter present, which combines with the available chlorin and renders it ineffective.

Brilliant green in 1:1,000 kills bacteria and protozoa alike nearly instantly. In 1:2,000 the protozoa are apparently unaffected, while the bacteria are killed in 20 minutes. This dye manifests its bactericidal power in about the same time in a dilution of 1:300, still not affecting the protozoa, while in 1:4,000 50 minutes is required to kill all the bacteria.

Copper sulphate in a dilution between 1:2,000-1:3,000 has no harmful effect on the protozoa, although there is a visible coagulation of the organic matter present, and the bacteria are found to be dead in 20 minutes.

The intestinal bacteria used frequently contained spore-forming varieties, and, while these could be demonstrated as the last to succumb in many cases, yet their resistance was much lower than that of the Giardia. These results are contrary to the old conception that the protozoa are more sensitive to antiseptics than are bacteria. The surprising fact was also noticed that in very dilute solutions of many of the antiseptics, the motility of the protozoa was greater and persisted for a longer period than in the controls. These observations were made so consistently that we regard them as evidences of a stimulating effect of low concentrations of otherwise toxic substances.

An Attempt to Grow Giardia Cuniculi in Pure Culture

By selecting an antiseptic less active against protozoa and choosing dilutions and time of exposure properly, it is possible to eliminate the bacteria from a mixture with protozoa, without apparently harming the latter. We attempted to use the material so treated as a starting point for growing Giardia in pure culture. The intestinal material and antiseptic, after being mixed for a length of time sufficient to kill the bacteria, was diluted 4 or 5 times with sterile broth, and then inoculated by a capillary pipet into tubes of Noguchi medium prepared with rabbit kidney, and (in a second attempt) with fragments of rabbit intestine. These materials were sterilized by discontinuous heat, and on other occasions by contact with dilute solutions of chloramin-T and brilliant green. The tubes thus inoculated were found nearly always to remain free from bacteria. The protozoa retained their motility for 2 or 3 hours. After an incubation for 3 or 4 days, characteristic cysts of Giardia were observed, but these disappeared finally and the method accordingly did not give the hoped-for results. Similar experiments were made on semi-solid mediums containing rabbit intestine which had been partially digested with trypsin and containing traces of added glucose. Our idea was to approximate as closely as possible in vitro the conditions obtained in the small intestine of the rabbit. These experiments were also failures, however, not even a development of cysts being observed.

Giardia have heretofore never been obtained free from bacteria and it has been often suggested that some protozoa are unable to live without the presence of bacteria, and herein lies a possible explanation for our failure. We are inclined, however, to believe the fault in these attempts has not been due to Giardia, for they behaved in a perfectly normal manner for several days, but rather to our inability in devising a suitable medium for the redevelopment of the cysts into motile forms. We intend to pursue the matter farther along these lines, if possible, with other parasitic protozoa.

Comparison of the Behavior of Giardia Cuniculi with Free Living Protozoa

Convinced of the difficulty of growing Giardia, we attempted to apply this method to the growth of free living protozoa, which are capable of development in comparatively simple mediums. The mixture of bacteria and protozoa (Ameba limax and a flagellate) were obtained from infusions of hay and cabbage, in which the protozoa were in symbiosis with many varieties of bacteria. As shown in Tables 2, 3, and 4, however, free living protozoa are

more susceptible and the accompanying bacteria less affected by the antiseptics used. Accordingly, in a dilution in which there was no difficulty in keeping the protozoa alive, it was possible to kill the bacteria. These bacteria are very sturdy saprophytic types, much more resistant than the intestinal flora. That free living protozoa show less resistance to the action of antiseptics than the parasitic types is again contrary to expectation, and is perhaps due to the comparatively simple chemical environment of the free living organism as compared to the parasite. It is certainly a state of affairs not paralleled in any sense by the bacteria, in which the saprophytic types are well recognized as the hardier.

CONCLUSIONS

In the intestinal content of the rabbit, it is possible to show that resistance of Giardia cuniculi against the action of certain antiseptic substances is markedly greater than the resistance of the intestinal bacteria. It is possible by making use of this difference in resistance to obtain Giardia free from all living bacteria, but attempts to subsequently grow them in pure culture have not succeeded.

Giardia are more resistant to the action of the toxic substances studied than are the free living protozoa studied.

A technical method has been developed which we believe will be useful in demonstrating the applicability of intestinal antiseptics against both the protozoa and the bacteria of the intestine.

ON THE NORMAL AGGLUTININS FOR DIFFERENT KINDS OF PATHOGENIC BACTERIA IN THE SERUM OF COLD-BLOODED ANIMALS

M. Takenouchi

From the Department of Hygiene and Bacteriology, University of Chicago

Normal agglutinins in animal serum for different kinds of bacteria have been studied extensively. Those in the serum of cold-blooded animals, however, seem to have been little investigated. In the literature, I could not find any particular article concerning this problem.

Following Professor Jordan's suggestion, I made a study of the serum of different species of turtle in their content of agglutinins against different kinds of bacteria, especially those which do not grow well at such low temperatures as those of cold-blooded animals.

The species of turtle tested are as follows: Chrysemys belli; Chrysemys elegans; Melacoclemmys lesueurii; Melacoclemmys (geographica?), and Chelydra serpentina (snapping turtle).

Two or three of each species were tested carefully. These were supplied by a firm in Chicago, between April 15 and 24, 1918. During the examination they were put in a box containing some tap water in the animal room of this laboratory.

The serum was taken from the arteria carotis without narcosis. Clear serum was separated and used for agglutination with different kinds of bacteria.

The following kinds of bacteria, stock cultures in this laboratory, were tested with the serum of normal turtles:

B. typhosus (Hopkins), B. paratyphosus A., No. 4; B. paratyphosus B., No. 12; B. coli communis; B. dysenteriae-Shiga; B. dysenteriae-Flexner; B. fecalis alcaligenes; B. pyocyaneus, and Staph. aureus I, II, III, and IV; also Staph. albus.

The usual technic of agglutination with small tubes was employed. The titer limit of agglutinins will be indicated in Table 1 by the dilution of serum in the tube in which slightly but decidedly positive agglutination was to be seen macroscopically. Many readings were made at different intervals, but only the one at the 6th hour incubation is noted in the table.

B. typhosus can be agglutinated by the normal serum of different species of turtle at a dilution of 20-60.

B. paratyphosus A can be agglutinated by the serum of Chrysemys belli at a dilution of 80.

B. paratyphosus B can be agglutinated by the normal serum of different species of turtle at a dilution of 20-40.

B. coli communis can be agglutinated by 40-80 times dilution of normal serum of Chrysemys elegans and Melacoclemmys lesueurii and (geographica?).

Received for publication May 15, 1918.

Shiga dysentery bacilli can be agglutinated by the normal serum of different species of turtle at 30-80 dilution, while Flexner dysentery bacilli are agglutinated by 160-240 dilution of those serums.

The normal serum of snapping turtle (Chelydra serpentina) shows a very high agglutinating value for Shiga bacilli (320-640), but a very low titer for Flexner bacilli.

B. fecalis alcaligenes can be agglutinated only slightly by the normal serum of different turtles.

B. pyocyaneus can be agglutinated by the normal serums of different species of turtle at dilution of 40-160. The rate of agglutination reaction of this bacillus is very rapid; usually within half an hour at incubation temperature the agglutination reaches the titer limit.

Different strains of Staph. aureus and albus can be agglutinated moderately by the normal serum of different genus of Chrysemys and Melacoclemmys at 20-40, or even 600-1200, times dilution. The serum of snapping turtle (Chelydra serpentina) shows a pretty high agglutinating value for several strains of staphylococcus (160-320 or even 640 times dilution).

TABLE 1

TESTS MADE WITH STOCK CULTURES OF BACTERIA AND SERUM OF DIFFERENT SPECIES OF TURTLES

	Chrysemys belli	Chrysemys elegans	Melaco-clemmys lesueurii	Melacoclem-mys (geographica?)	Chelydra serpen-tina
B. typhosus	20	20-40	60	20-40	20
B. paratyphosus A	80	40	30	10	20
B. paratyphosus B	40	20	30	20-40	40
B. coli communis	10	40-80	60	80	40-80
B. dysenteriae Shiga	40	40-80	30	80	320-640
B. dysenteriae Flexner	Not tested	160	240	160	20
B. fecalis alcaligenes	10	20	Not tested	10	Not tested
B. pyocyaneus	40-80	80-160	40	20	Not tested
Staph. aureus I	320	1,200	960	1,200	320
Staph. aureus II	Not tested	40	40	40	160
Staph. aureus III	Not tested	20-40	40	20	640
Staph. aureus IV	Not tested	20-40	40	20	320
Staph. albus	Not tested	20-40	40	10	80

As far as I am aware, "normal agglutinins" are not present in new-born warm-blooded animals,[1] and infants show lower normal agglutinating values than adults. It has been shown that living bacteria may enter the lymphatic and portal circulation from the intestine while the individual is apparently in perfect health. According to the prevailing opinion it is a possible and not unreasonable supposition that the presence of "normal agglutinins" is due to slight unrecognized infection with some specific bacilli or to the absorption of specific toxic substances from the intestinal canal.

It would be of considerable interest, therefore, to determine the agglutinating values of the serum of young cold-blooded animals (for

[1] Savage, W. G.: Jour. Hyg., 1918, 17, p. 34.

instance, turtles) at different intervals for different kinds of pathogenic bacteria. Unfortunately, material for this purpose has not been available.

Since I have not tested the serum of very young cold-blooded animals, I am unable to give a definite opinion, but it does not seem probable that those agglutinins in cold-blooded animals for different kinds of pathogenic bacteria, which grow usually with great difficulty at such a low temperature as the body temperature of cold-blooded animals, are due to slight unrecognized infection with some specific bacteria or to the absorption of some toxic substance from the intestinal canal.

CONCLUSIONS

1. The normal serums of Chrysemys belli, Chrysemys elegans, Melacoclemmys lesueurii and Melacoclemmys (geographica?) show fairly high agglutinating values for certain bacteria which are pathogenic for warm-blooded animals and do not grow readily at the body temperature of cold-blooded animals.

2. These facts are theoretically interesting, because, according to the prevalent hypothesis, the occurrence of normal agglutinins is due to a slight unrecognized infection or to the absorption of some specific toxic substance from the intestinal canal during life. This seems improbable, especially in the case of cold-blooded animals.

CYTOLYTIC ACTION OF NORMAL AND IMMUNE SERUM ON INFUSORIA

M. Takenouchi

From the Department of Hygiene and Bacteriology, University of Chicago

INTRODUCTION

The normal occurrence in the blood of various animals of bodies destructive to the cells of other animal species is generally recognized. It has been shown, for instance (H. Windsor), that a substance hemolytic for guinea-pig corpuscles occurs in human serum. We know also that fresh serum of many, if not all, animals contains a toxic substance, which causes contraction of smooth muscle; this toxic property diminishes considerably when the serum is kept and has nothing to do with the anaphylactic reaction.[1]

We have found no systematic observation concerning the action of "cytolytic" or toxic substance or substances in normal serums of various animals on different kinds of infusoria. Rössle,[2] however, made some observations on the production of antibodies to infusoria, and in the course of this work noted a few facts regarding the action of normal serum on paramecium. He compared the action of a 5-10 times dilution of normal and specific immune serums on paramecia, and found intensive paralyzing of paramecia to be the specific toxic action, but he did not find any definite phenomena which could be regarded as analogous to bacteriolysis. It must be especially noted here that Rössle used for the dilution of the normal and immune serums the liquid of his paramecia-culture, which was prepared without adding any particular electrolytes (salts).

The present work was designed principally to investigate the influence of normal animal serums on the monocellular organisms, such as protozoa, which are large and easily distinguishable whether they are active or dead.

METHOD AND TECHNIC EMPLOYED

Clear active or heated serums were diluted and distributed in series so as to determine the smallest amount capable of killing about the same number of infusoria in a definite time. Using small test tubes, a series of several dilutions of serum with 0.6% sodium chlorid solution was made. To each dilution of serum an equal amount of paramecia suspension in water was added. The time

Received for publication May 15, 1918.

[1] Bayliss, W. H.: Principles of General Physiology, 1915, p. 730.

[2] Arch. f. Hyg., 1905, 54, p. 1.

of this addition was noted, and after 10, 15 and 60 minutes or 24 hours, the paramecia in tubes were microscopically examined to note their movement and any alteration in their form. Usually the tubes were incubated at 34-35 C., but in a few instances were kept at room temperature.

By means of a small capillary pipet, a small amount of liquid containing the protozoa was withdrawn from the lower layer, much care being taken not to destroy paramecia with the end of the pipet. One to two drops of this liquid were put on a slide and the activity of motion, morphologic alterations of paramecia, such as vacuoles, swelling of body, discharge of trichocysts and disintegration of the protoplasm, were microscopically observed with a Spencer objective 16 mm. and 4 mm. and ocular 10 X.

The culture of paramecia (Paramaecium caudatum) was obtained from Dr. Libble H. Hyman, Hull Zoological Laboratory of the University of Chicago, who kindly gave me many suggestions about the methods of infusoria culture. The culture was grown as follows:

Hay infusion, diluted with tap water, was sterilized with some hay. In a sterile jar containing about 400 c c of the diluted infusion, I transfered several cubic centimeters of the original paramecia culture, containing several kinds of bacteria on which the paramecia feed. This was kept at room temperature between 21 and 24 C., sometimes at incubator temperature (Rössle). The paramecia may appear abundantly in a week after starting the culture and can be used for the present purpose.

Three strains of paramecia (Paramaecium caudatum) were used as follows: (1) Paramecia alone, without any other kinds of infusoria in the culture; (2) a culture of paramecia with a small number of colpoda, and (3) a paramecia culture with very small number of stylonichyia.

Suspension of paramecia: Instead of using the culture liquid itself for the experiments I used paramecia suspension in water made as follows: a heavy paramecia culture is poured into a large, sterile, narrow-mouth flask, containing a sufficient amount of sterilized tap water, bringing the surface of the water very close to the neck of the flask. Within a few minutes almost all the paramecia come up to the surface and can be collected easily with a clean pipet. Repeating this process several times, using each time a fresh sterile flask with sterilized tap water at room temperature, any other substances in the original culture liquid which might affect in any way the action of the substances in the serum on paramecia can be almost entirely eliminated.

To count the number of paramecia contained in a suspension, 1 c c or more was mixed with 10 c c of about 20% pure gelatin and plated in a Petri dish in which the number of animals can be counted just as colonies of bacteria are counted.

The number of paramecia in a suspension can be counted quite easily in a narrow tube (a thin walled 1 c c pipet graduated in 100ths), holding the pipet horizontally and closing the upper end by a rubber bulb. The number of paramecia swimming in the liquid can be determined very easily, if there are not too many animals. If the protozoa are too numerous the suspension may be diluted with water. Throughout this work I used a suspension of from 100 to 120 paramecia in 1 c c of water.

In the following tests with older serums microscopic examination and cultivation with agar agar plate were made each time to avoid any error which might be caused by the use of spoiled or contaminated serum. Because of the strong injurious action of all kinds of disinfectants on paramecia (swelling, discharge of trichocysts and disintegration) none of them was used for the purpose of keeping the serum.[3]

[3] Kölsch: Zoolog. Jahrbücher, 1902, 16, p. 273.

SALTS AND THEIR TOXICITY AGAINST PARAMECIA

The great significance of the kinds of salts and their concentration in biologic experiments does not need any further explanation.[4, 5]

All serologic reactions are dependent to a great extent on the presence of electrolytes in the medium. Calcium sulphate and barium sulphate interfere with the action of complement (Hektoen and

TABLE 1*

RESULTS OBTAINED IN THE EXAMINATION OF THE INDIVIDUAL SALTS

		1	2	3	4	5	6	7
M/5 salt solution in distilled water		0.5	0.4	0.3	0.2	0.15	0.1	—
Distilled water		—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia		0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Time of Observation							
$MgSO_4.7H_2O$	15 minutes	+	++	+++	+++	+++	+++	+++
	30 minutes	†	†	++	+++	+++	+++	+++
	60 minutes	†	†	†	+++	+++	+++	+++
	20 hours	†	†	†	+++	+++	+++	+++
NH_4Cl	15 minutes	†	+†	++	+++	+++	+++	+++
	30 minutes	†	†	+†	+++	+++	+++	+++
	60 minutes	†	†	+†	++	+++	+++	+++
	20 hours	†	†	†	†	+†	++	+++
$BaCl_2$§	17 minutes	†	†	†	†	†	†	+++
KBr	15 minutes	†	+†	++	+++	+++	+++	+++
	30 minutes	†	+†	++	+++	+++	+++	+++
	60 minutes	†	†	++	+++	+++	+++	+++
	20 hours	†	†	†	+†	+++	+++	+++
NaBr	15 minutes	†	+†	+†	+++	+++	+++	+++
	30 minutes	†	†	+†	+++	+++	+++	+++
	60 minutes	†	†	†	++	+++	+++	+++
	20 hours	†	†	†	+	+++	+++	+++
NaI	15 minutes	†	+++	+++	+++	+++	+++	+++
	30 minutes	†	++	+++	+++	+++	+++	+++
	60 minutes	†	†	++	+++	+++	+++	+++
	20 hours	†	†	†	+++	+++	+++	+++
NaCl	15 minutes	†	+	+++	+++	+++	+++	+++
	30 minutes	†	+†	++	+++	+++	+++	+++
	24 hours	†	†	++	+++	+++	+++	+++

* +++ all animals motile. ++ some of them are motile. + very few of them are motile, the others are nonmotile. +† almost all of the animals are preeipitated and very few of them are slowly motile. † all animals are preeipitated and nonmotile.

§ This result indieates that Ba salt is exeeedingly poisonous for the infusoria, as many investigators have stated.

Ruediger). Magnesium sulphate, calcium chlorid, potassium iodid, sodium iodid and barium chlorid are especially unfavorable for the complement action (Friedberger, v. Dungern and Coca, Pribram). According to Gengou, sodium citrate checks the complement action to

[4] Loeb, J.: Pflüger Arch., 1903, 97, p. 394.

[5] Loeb, J., and Wasteny, Hardolph: Biochem. Ztschr., 1911, 32, p. 155.

some extent. Recently Noguchi studied this phenomenon systematically.[6]

Paramecium itself does not require any particular salt to be added to the medium in which the animal is living; but these animals must be put into a dilution of serum and as we shall see later, the toxic action of normal serum on paramecia depends very much on the salt present. Here we have to choose salts and a concentration, which shall not be injurious to these animals and which shall be favorable to the action of serum on paramecia.

The following salts were examined individually with their M/5 solution with respect to their action on paramecia: $MgSO_4.7H_2O$; NH_4Cl; $BaCl_2$; $NaBr.2HO$; NaI and NaCl.[7]

Altho the microscopic examination was made in each case in the experiments previously described, only the result of M/5 NaCl will be given, since the others are more or less similar to this.

Microscopic Examination:

1. Tube 1: 7 minutes after the addition of suspension of paramecia. All animals are flat, have many vacuoles in their bodies. They are, however, all actively swimming around; no marked discharge of trichocysts. The anterior end of the body is injured most.

2. Tube 2: 17 minutes. Almost the same picture as above.

3. Tube 1: 30 minutes. Many animals have 2 large vacuoles and numerous smaller ones in their bodies. Their bodies markedly flattened. The cilia on the surface of the anterior half of the body are almost motionless. Trichocyst discharge is not marked.

4. Tube 2: 33 minutes. All animals are swollen. Some of them have discharged trichocysts around themselves.

5. Tube 3: 35 minutes. All animals are swimming around very actively, with number of small vacuoles in their body. No other remarkable change.

From this experiment with M/5 NaCl solution, we see that NaCl is less toxic than other salts, and 0.3 c c (Tube 3) of M/5 NaCl solution is only slightly injurious to paramecia. Repeated tests with various concentrations of this salt have shown that 0.6% solution is the rational concentration for our present purpose, that is, the solution of NaCl of that concentration, diluted again to two by the suspension of paramecia, is not injurious to paramecia at all, and, at the same time it does not depress the action of normal serum on paramecia. Therefore the 0.6% solution of NaCl was used for the dilution of serum every time. In each titration 0.5 c c of serum dilution with 0.6% NaCl solution and 0.5 c c of suspension of paramecia in water were used; the approximate salt concentration in every tube was about 0.3%.

[6] Biochem. Ztschr. 1907, 6, p. 172.

[7] Lösch, Karl: Zoolog. Jahrbücher, 16, 1902, p. 357.

A. ACTION OF NORMAL SERUMS OF WARM-BLOODED ANIMALS ON PARAMECIA

(*a*) Human Serum: Human blood was taken just before lunch in 6 tubes each containing 5-6 c c blood.

Two series of serum dilution were tested 3 hours later, one at room temperature, the other in the incubator for 1 hour and then at room temperature. As in many other cases the human serum acts on paramecia very much quicker at incubator temperature than at room temperature. At the end of 1 hour the first examination was made, and after that time both series were put at room temperature for about 20-24 hours, when the second examination was made.

TABLE 2

Results Obtained in the Tests with Human Serum

	1	2	3	4	5	6
Active human normal serum, diluted with 0.6 % NaCl solution to 1 : 5....	0.4	0.3	0.2	0.15	0.1	—
0.6 % NaCl solution....................	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia..............	0.5	0.5	0.5	0.5	0.5	0.5
Series 1.—At room temperature:						
Time of observation—						
30 minutes..........................	+†	++	+++	+++	+++	+++
45 minutes..........................	†	+†	+†	+++	+++	+++
20 hours............................	†	†	†	+†	+++	+++
Series 2.—At the incubator for 1 hour, then at room temperature:						
Time of observation—						
30 minutes..........................	+†	+†	+†	+++	+++	+++
45 minutes..........................	†	†	+†	+†	+++	+++
20 hours............................	†	†	†	+†	+++	+++

Microscopic examination at the end of one hour.

Series 1: Observation at room temperature.

Tube 1: Very little disintegration and discharge of trichocyst. Some are still motile, but swollen.

Tube 2: Some animals are swollen, and slowly motile.

Tube 3: All animals are actively motile.

Series 2. Observed after 1 hour in the incubator.

Tube 1: All animals are disintegrated; trichocysts discharged. Colpoda are still very actively swimming around.

Tube 2: The picture is similar to Tube 1.

Tube 3: Some animals are in active locomotion, but move very slowly. Few of them are already disintegrated. Others are swollen, with trichocysts discharged. Colpoda still alive in active condition.

Microscopic examination of both series after 24 hours at room temperature shows same result.

1. Tube 1: Very few visible residues of disintegrated paramecia. Even single colpoda not to be seen.

2. Tube 2: Same as Tube 1.

3. Tube 3: Very few paramecia in disintegrated condition. Colpoda are very actively swimming around in the medium.

4. Tube 4: Almost all paramecia are disintegrated. Very active locomotion of colpoda.

5. Tube 5: Paramecia actively motile, with many vacuoles. Some of them are already disintegrated. Many colpoda are very actively swimming around.

6. Tube 6: All paramecia and colpoda are in active condition.

Colpoda which were simultaneously cultivated with paramecia and came into the suspension, are more resistant against human blood serum than paramecia.

The titer of fresh human blood serum lies between Tubes 3 and 4; that is, between 0.2 and 0.15 c c of 1 : 5 dilution.

The blood serum specimen kept at room temperature lost its activity more rapidly than that kept in the ice-box.

The titration of another serum specimen taken on Jan. 12, 1918, 2 hours after luncheon, showed that there was no definite relation between the amount of the toxic substance in the blood at different periods.

The human serum heated for one-half hour at 56 C. was tested in the same manner as before. After 24 hours all animals in the medium in every tube were swimming around actively; there was no sign of toxic action of inactivated serum on infusoria.

TABLE 3

RESULTS OBSERVED IN THE TESTS WITH SERUMS FROM DIFFERENT ANIMALS

Serum	Titer against the Same Number of Paramecia	
	Dilution	Amount, c c
Human	1 : 5	0.2 : 0.15
Horse	1 : 5	0.2 : 0.15
Sheep	1 : 5	0.15 : 0.1
Beef	1 : 20	0.2 : 0.15
Hog	1 : 20	0.4 : 0.3
Guinea-pig*	1 : 20	0.3 : 0.2
Rabbit	1 : 20	0.3 : 0.2
Pigeon	1 : 10	0.2 : 0.1

* I noted some individual variation.

As in the case of carbolic acid test, paramecia, acted on by human serum, discharge trichocysts[8] in the medium. These can be seen distinctly under the low power of the microscope.

Several normal human serums were obtained and titrated with almost the same result. Each specimen was carefully tested for Wassermann reaction with negative result.

There is no difference in resistance against normal serum between paramecia raised at room temperature and those cultivated at incubator temperature, if, in making the suspension, care is taken to avoid sudden changes of temperature of the medium.

Human normal spinal fluid, which is said to have no complement (MacKenzie and Martin, Houston, Hektoen) was tested in the same way with the infusoria, but no sign of toxic action was observed.

[8] The discharge of trichocysts under the influence of stimuli has been studied by Massart (1901) and by Statkewitsch (1903). Crushing the animal causes discharge of trichocysts in the region of injury. Weaker mechanical stimuli do not have this effect. If the animal is heated rapidly till it is killed, it discharges the trichocysts before dying; if heated slowly, this effect is not produced. Neither cold nor increased pressure have any effect on trichocysts. Many chemicals, particularly acids, produce the discharge. An electrical induction shock causes the same effect; when it is weak, the discharge is at the anode only; stronger shock causes discharge at both anode and cathode; still stronger shock causes discharge of trichocysts over the entire surface of the body (Statkewitsch 1903). (Jennings: Behavior of Lower Organisms, p. 89.)

(*b*) Serums of Other Warm-Blooded Animals: Using the same method normal serums of different warm-blooded animals were tested with the results given in Table 3.

In all tested serums of warm-blooded animals the activity against paramecia is entirely destroyed when they are heated at 56 C. for 20 minutes. For reactivation refer to Section 6. The microscopic alteration in the body of the protozoa is just the same as that observed in the case of human serum.

B. ACTION OF SERUM OF COLD-BLOODED ANIMALS

(*a*) Frog Blood Serum: Blood was taken direct from the heart. After centrifugalization clear serum separated, which was titrated as usual.

TABLE 4

Tests Made with Frog Blood Serum

	1	2	3	4	5	6	7
Active normal frog serum diluted with 0.6 % NaCl to 1 : 10	0.5	0.4	0.3	0.2	0.15	0.1	—
0.6 % NaCl solution	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation at room temperature:							
10 minutes	†	+†	+	++	++	++	+++
30 minutes	†	†	†	†	†	+†	+++

Microscopic examination after 1 hour and 25 minutes.

Tube 3: Disintegration; spherical shape; very marked trichocyst discharge. Two very large vacuoles in the body of other animals which are about to disintegrate.

Tube 4: Nonmotile; few are already disintegrated. Most of them are swollen and have a spherical shape, with two very large vacuoles in the body. Trichocyst discharge very marked.

TABLE 5

Showing the Slight Individual Variation in the Activity of Normal Frog Serum on Paramecia

	1	2	3	4	5	6	7
Frog 5:							
Dilution of serum 1 : 50	0.5	0.4	0.3	0.2	0.15	0.1	—
Time of observation at room temperature—							
30 minutes	†	†	†	+†	++	+++	+++
60 minutes	†	†	†	†	++	+++	+++
18 hours	†	†	†	†	+	+++	+++
Frog 6:							
Time of observation at room temperature—							
30 minutes	†	†	+†	++	+++	+++	+++
60 minutes	†	†	+†	++	+++	+++	+++
18 hours	†	†	†	+†	+	+++	+++

Tube 5. All animals are entirely nonmotile. More or less swollen, but no disintegration to be seen.

Tube 6. Very slight motion of cilia in the cystostom still present.

A slight individual variation in the strength of action on paramecia of normal frog serum is recorded in Table 5. Blood was taken and tested on March 20, 1918.

The activity of normal frog serum on paramecia is entirely destroyed when the serum is heated at 56 C. for 20 minutes.

The blood serum of normal frog contains hemolysin against the corpuscles of rabbit.

Given corpuscles or protozoa succumb to the dissolving effects of the complement and lytic substance in the frog serum at different rates which cannot be directly compared with each other from the result obtained in each experiment, because the relation of the number of cells to the dissolving agencies is not the same in the two cases. In other words, we use about 0.5 c c of paramecia suspension, 1 c c of which contains about 100-120 protozoa, while in the hemolysis, we use 0.5 c c of 5% suspension of corpuscles in salt solution.

(*b*) Turtle Blood Serum (Chrysemys Picta): The blood was taken from arteria carotis of the turtle. Clear serum was titrated in the usual way, 0.6% NaCl solution being used for the dilution. The titer limit lies between 0.15 and 0.1 c c of 1 : 50 dilution. The clear serum was heated for 30 minutes to a temperature varying from 45-60 C., after which the activity of the serum was again noted.

TABLE 6

RESULTS OF TESTS MADE WITH TURTLE BLOOD SERUM

		1	2	3	4	5	6	7	8
Heated serum	Dilution 1 : 5....	0.5	0.4						
	Dilution 1 : 10...			0.5	0.4	0.3	0.2	0.1	—
	0.6 % NaCl......	—	0.1	—	0.1	0.2	0.3	0.4	0.5
	Suspension of paramecia....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Time of Observation at 23 C.								
Heated at 45 C. for 30 minutes	7 minutes......	†	†	+†	+†	+++	+++	+++	+++
	15 minutes......	†	†	†	†	+	+†	+	+++
	30 minutes......	†	†	†	†	†	†	†	+++
Heated at 50 C. for 30 minutes									
	30 minutes......	+++	+++	+++	+++	+++	+++	+++	+++
	60 minutes......	+++	+++	+++	+++	+++	+++	+++	+++
	2 hours.........	+++	+++	+++	+++	+++	+++	+++	+++
	18 hours.........	†	+†	+†	++	+++	+++	+++	+++
Heated at 53 C. for 20 minutes									
	30 minutes......	+++	+++	+++	+++				
	4 hours.........	+++	+++	+++	+++				
	24 hours.........	+++	+++	+++	+++				

According to these results heating at 50 C. for 30 minutes is not sufficient to cause a complete inactivation of the complement against paramecia in the normal turtle serum; for this it is necessary to heat the serum at 53 C. for 20 minutes.

Repeated titration with dilution of 1 : 50 shows that by heating the turtle serum at 45 C. for 30 minutes, the complement in the serum is affected only a little; the titer on paramecia is almost the same as the original serum*

*The turtle serum contains an appreciable amount of natural hemolysin against rabbit corpuscles. A more detailed description will be given in a subsequent paper.

ADSORPTION TEST

As stated by v. Dungern, Landsteiner and Eisner,[9] Andrejew[10] and other investigators, substances in blood serums can be adsorbed by many kinds of adsorbents, such as casein, quartz sand, coal, barium sul-sulphate, kaolin, etc.

1. *Adsorption by Bone Black.*—Five c c normal serum was mixed with 0.5 gm. charcoal and after shaking thoroughly in a tube, was put in the incubator for 1 hour at 36 C. The serum was then centrifugalized for about 10 minutes and the clear serum used in titration. As a control experiment, the same amount of serum was kept in the same incubator for the same time and centrifugalized and titrated in the same manner.

(*a*) Adsorption of horse serum by bone black. The serum was kept for 48 hours in the ice-box. For the dilution of the serum 0.6% NaCl solution was used.

TABLE 7

RESULTS OF TESTS MADE WITH BONE BLACK

	1	2	3	4	5	6	7
Adsorbed horse serum diluted to 1 : 5 with 0.6 % NaCl solution........	0.5	0.4	0.3	0.2	0.15	0.1	—
0.6 % NaCl solution..........	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia....	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
20 minutes.............	†	++	+++	+++	+++	+++	+++
60 minutes.............	†	+†	++	+++	+++	+++	+++
20 hours...............	†	+†	+	++	++	+++	+++
Control serum:							
20 minutes.............	†	+†	++	+++	+++	+++	+++
60 minutes.............	†	†	†	+++	+++	+++	+++
20 hours...............	†	†	†	++	+++	+++	+++

(*b*) Adsorption of beef serum by bone black. The serum was kept for 48 hours in the ice-box.

The titer of the adsorbed beef serum is between 0.3 and 0.2 c c of 1:20 dilution, while that of the control experiment is between 0.15 and 0.1 c c of 1:20 dilution of normal beef serum.

2. *Adsorption by Infusorial Earth.*—Five c c of 10% suspension of infusorial earth in distilled water and 5 c c of normal beef serum, 120 hours old, were shaken thoroughly and the mixture put in the incubator for an hour and then centrifugalized for about 10 minutes. This clear serum, being diluted already to 1:1 with distilled water, ought to be diluted further to 1:20 with salt solution and the final salt concentration should be 0.6 % NaCl.

The adsorbents used were labeled chemically pure, and repeated experiments showed no substances present that were injurious to the infusoria.

[9] Wiener klin. Wchnschr., 1904, 24, p. 676.

[10] Arbeit a. d. k. Gesndthsamte, 1909, 33, p. 84.

TABLE 8

RESULTS OF TESTS MADE WITH INFUSORIAL EARTH

	1	2	3	4	5	6	7
Adsorbed normal beef serum 1 : 20 dilution with 0.6 % NaCl..........	0.5	0.4	0.3	0.2	0.15	0.1	—
After 20 hours..............	†	†*	+++	+++#	+++	+++	+++

* Denotes the position of the titer limit of the adsorbed serum, while # denotes that of control test with normal beef serum without any adsorption. A marked difference between these two titer limits is noted.

Owing to the low titer of normal serum it was not possible to determine any mathematical relation concerning the law of adsorption in modern interpretation.

These results show that the toxic substance or substances which act on paramecia can be adsorbed by some adsorbents.

REACTIVATION

In each case of titration of normal serum, the latter loses the greater part of its toxic properties by heating at 56 or 53 C. for half an hour.

In adding normal guinea-pig serum for reactivation, we must keep in mind the fact that the normal serum of guinea-pig itself exerts a strong toxic action on paramecia.

1. *Reactivation of Inactivated Fresh Normal Beef Serum by Fresh Normal Guinea-Pig Serum.*—A very small amount of normal active beef serum, for instance, 0.15-0.10 c c of 1 : 20 dilution, is still capable of causing some paramecia to disintegrate. Assuming that the fresh beef serum has as much complement as the fresh guinea-pig serum, the reactivation test of inactivated beef serum was made.

TABLE 9

TESTS MADE WITH INACTIVATED BEEF SERUM

	1	2	3	4	5	6	7
Inactive beef serum diluted with 0.6 % NaCl to 1 : 20...	0.4	0.3	0.2	0.15	0.1	0.5	—
0.6 % NaCl solution.........	—	0.2	0.2	0.25	0.3	—	0.3
Guinea-pig serum diluted to 1 : 20......................	0.1	0.1	0.1	0.1	0.1	—	0.2
Suspension of paramecia in water...................	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
1 hour................	†	†	†	†	†	+++	+++
24 hours...............	†	†	†	†	†	+++	+++

Microscopic examination at the end of 1 hour gave a uniform result in each tube except the control test (Nos. 6 and 7); that is, all animals are entirely disintegrated. The result of this experiment is very remarkable and can be easily understood as in the case of bacteriolysis and hemolysis.

2. *Reactivation of Inactive Hog Serum by Fresh Normal Guinea-pig Serum.*—Fresh active hog serum in 0.1 c c of its 1 : 20 dilution is capable of diminishing the free mobility of paramecia and the inactive hog serum has no toxic action

on paramecia. I tried to test the possibility of reactivation of inactivated hog serum by the aid of active normal guinea-pig serum, with a distinctly smaller amount of the latter than the titer limit against paramecia, which is estimated by direct titration.

TABLE 10

TESTS MADE WITH INACTIVE HOG SERUM

	1	2	3	4	5	6	7
Inactive hog serum diluted with 0.6 % NaCl solution to 1 : 20....................	0.4	0.3	0.2	0.15	0.1	0.5	—
0.6 % NaCl solution..........	—	0.1	0.2	0.25	0.3	—	0.3
Active normal guinea pig serum diluted with 0.6 % NaCl to 1 : 20..............	0.1	0.1	0.1	0.1	0.1	—	0.2
Suspension of paramecia in water....................	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
60 minutes............	+†	+†	+†	+†	+†	+++	+++
2 hours..............	†	†	†	†	†	+++	+++
24 hours................	†	†	†	†	†	+++	+++

This fresh hog serum alone in 0.1 c c of 1 : 20 dilution is not sufficient to destroy all paramecia: and this is due, at least to some extent, to a relatively insufficient amount of complement in it, while the inability of 0.1 c c of 1 : 20 dilution of fresh guinea-pig serum to disintegrate the same number of paramecia, is due to a relatively insufficient amount of lytic substance (amboceptor, which is thermostabile) in the normal guinea-pig serum.

The last statement, that in the guinea-pig serum the lytic substance (normal amboceptor) is insufficient in amount to combine with the whole amount of complement, is proved by the experiment described in Table 11.

TABLE 11

TESTS PROVING THAT IN GUINEA-PIG SERUM THE LYTIC SUBSTANCE IS INSUFFICIENT IN AMOUNT TO PROPERLY COMBINE

	1	2	3	4	5	6	7	8	9
Guinea-pig serum diluted to 1 : 20 with 0.6 % NaCl.............	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.2	0.3
0.6 % NaCl solution......	—	0.05	0.1	0.15	0.2	0.25	0.35	0.3	0.2
Inactive beef serum diluted with 0.6 % NaCl solution to 1 : 50.......	0.35	0.3	0.25	0.2	0.15	0.1	—	—	—
Suspension of paramecia..................	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hour in the incubator and then at room temperature):									
60 minutes.........	+†	+†	++	++	+++	+++	+++	+++	+++
16 hours...........	†	†	†	†	†	+	++	+++	+++
17 hours...........	D	D	D	D	D—				

Microscopic examination at the end of 17 hours. D denotes entire disintegration of paramecia; D— denotes incomplete disintegration of paramecia.

While 0.15 c c of 1:20 dilution of fresh normal guinea-pig serum is unable by itself to kill the paramecia, the same amount of fresh normal guinea-pig serum mixed with a sufficient amount (over 0.15 c c of 1:50 dilution) of inactive beef serum, containing normal amboceptor only, is able to kill the paramecia entirely; in other words, the insufficient amount of normal amboceptor needed to fix the corresponding amount of complement present in the fresh guinea-pig serum is supplied in this case by the inactivated beef serum.

5. *Reactivation of Inactive Rabbit Serum by Fresh Normal Guinea-Pig Serum.*—I observed a phenomenon which seems to be analogous to the "complement deviation," in the estimation of normal amboceptor in normal rabbit serum, as seen in Table 12:

TABLE 12

DETAILS REGARDING A PHENOMENON OBSERVED DURING THE TESTS

	1	2	3	4	5	6	7	8	9	10	11
Inactive rabbit serum diluted with 0.6 % NaCl to 1 : 5	0.4	0.3	0.2	0.15							
To 1 : 10					0.3	0.2	0.15				
To 1 : 20								0.3	0.2	0.15	—
0.6 % NaCl	—	0.1	0.2	0.25	0.1	0.2	0.25	0.1	0.2	0.25	0.5
Fresh normal guinea-pig serum 1 : 20	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	—
Suspension of paramecia in water	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation:											
1 hour	+++	+++	+++	+++	+++	†	†	†	+	++	+++
20 hours	+++	+++	++	++	†	†	†	†	†	+	+++

The titer limit of normal guinea-pig serum used in this test as the source of complement is between 0.2 and 0.15 c c of 1:20 dilution; 0.1 c c of that dilution is certainly incapable of killing the number of paramecia present within 20 hours.

This examination was repeated several times with different serums and the result was always the same. In all tubes in which the amount of amboceptor is in excess, there is no action of the serum on paramecia, a phenomenon analogous to the deviation of complement according to Neisser and Wechsberg.

The same "prozone" is observed also when immune rabbit serums are used instead of normal serum; but, contrary to our expectation, the extent of the "prozone" is not parallel with the amount of immune amboceptor in the immune serum; that is, the difference in the extent of the "prozone" of normal and immune serum is rather faint. We expect to discuss this matter more fully from the point of view of the theory of complement deviation. In our case "agglutination" can not be considered as a cause of this phenomenon as it is thought to be by some authors in the case of bacteriolysis,[11] because we do not see any sign of agglutination of paramecia in any one of the tubes in the "prozone" of this experiment.

4. *Reactivation Test of the Inactive Turtle Serum by the Normal Guinea-Pig Serum.*—Normal turtle serum was inactivated by heating at 53 C. for 20 minutes in a water bath. Normal guinea-pig serum was used as the source of complement.

[11] Buxton: Jour. Med. Research, 1905, 13, p. 431.

TABLE 13*

TESTS MADE WITH INACTIVE TURTLE SERUM

	1	2	3	4	5	6	7	8	9	10
Inactive turtle serum, diluted with 0.6 % NaCl to 1 : 5	0.4	0.3								
To 1 : 10			0.4	0.3						
To 1 : 20					0.4	0.3				
To 1 : 50							0.4	0.3		
To 1 : 100									0.4	0.3
0.6 % NaCl	—	0.1	—	0.1	—	0.1	—	0.1	—	0.1
Guinea-pig serum diluted with 0.6 % NaCl to 1 : 30§	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0 .1
Suspension of paramecia	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
After 24 hours	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

* The control test tubes are omitted.

§ The smallest amount of normal guinea-pig serum capable of killing a given number of paramecia in a definite length of time was previously determined in the usual way. The amount used in this case is about one-half of the titer limit.

After 2-4 hours all the animals in all the tubes were swimming around vigorously; no marked sign of toxic action. Probably the normal amboceptor in the turtle serum is unable to be complemented by the normal complement in the guinea-pig serum. The normal hemolytic amboceptor against rabbit corpuscles in the turtle serum is not complemented at all by the normal guinea-pig serum.[12]

5. *The Lytic Amboceptor Against Paramecia in the Inactivated Normal Frog Serum.*—The lytic amboceptor against paramecia tested under these conditions was also unable to be reactivated by the addition of normal guinea-pig serum.

6. *Reactivation of Inactive Turtle Serum with Normal Frog Serum.*—The reactivation of inactive turtle serum by the normal active frog serum was attempted, but positive complementation was not observed. The hemolytic amboceptor in the normal turtle serum, which will be fully discussed in the subsequent paper, is also unable to be complemented by the normal complement of frog serum.[13]

SALT CONCENTRATION AND THE ACTION OF SERUM

As in other serologic reactions salt concentration of the medium is of great importance in the toxic action of normal serum on paramecia. Too high salt concentration and too low electrolyte concentration are both unfavorable for the toxic action of normal serum on paramecia, as indicated by the results of the following experiment.

Three series of tubes containing different dilutions of serum with different amounts of salt are compared in their titer of toxic action on paramecia.

In this case there is no sign of toxic action of beef serum; all animals in all tubes are active even after 47 hours at room temperature.

[12] Ritz: Ztschr. f. Immunitätsforsch., 1911, 9, p. 321.

[13] Noguchi, H.: Bull. Univ. of Pa., 1902, 15, p. 301.

TABLE 14

SALT CONCENTRATION TESTS. SERIES 1

	1	2	3	4	5	6	7
Beef serum diluted with distilled water to 1 : 20.......	0.5	0.4	0.3	0.2	0.15	0.1	—
1 c c of 0.85 % NaCl diluted with distilled water to 1 : 20......................	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia in water......................	0.5	0.5	0.5	0.5	0.5	0.5	0.5
After 2 - 4 hours...........	+++	+++	+++	+++	+++	+++	+++

TABLE 15

SALT CONCENTRATION TESTS. SERIES 2

	1	2	3	4	5	6	7
Beef serum diluted with 0.3 % NaCl solution to 1 : 20..	0.5	0.4	0.3	0.2	0.15	0.1	—
1 c c of 0.85 % NaCl diluted with 0.3 % NaCl solution to 1 : 20...................	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia in water......................	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
20 minutes.............	+†	+†	++	+++	+++	+++	+++
40 minutes.............	†	†	†	†	+++	+++	+++
60 minutes.............	†	†	†	†	+++	+++	+++
20 hours................	†	†	†	†	+++	+++	+++

TABLE 16

SALT CONCENTRATION TESTS. SERIES 3

0.6 % NaCl solution is used instead of 0.3 % in the test in Series 2.							
20 minutes...................	†	†	+†	+++	+++	+++	+++
40 minutes...................	†	†	†	+†	+++	+++	+++
60 minutes...................	†	†	†	†	+++	+++	+++
20 hours......................	†	†	†	†	+†	+++	+++

The microscopic examination of the second and third series 1 hour after the addition of the suspension of paramecia shows almost the same degree of morphologic alteration of paramecia.

No sign of toxic action is apparent in the first series, where the salt concentration is low. In the second series the result of the serum action is almost the same as in the third series, where the salt concentration is as high as in many other series of experiments with normal serum on paramecia. A slight difference in the rapidity of action between the two series is noted, the action in the second series being somewhat slower than that in the third. Our result agrees exactly with that obtained by Buchner in his work on the bactericidal substance of normal serum. According to his findings, this can be destroyed by dilution of serum over 12 times with water and by dialysis against water (not against salt solution), and it can be reduced again by addition of salt solution.

Using the paramecia-immune rabbit serum, we obtained the same results, which indicates the necessity of electrolyte for the action of amboceptor and complement on paramecia.

The negative result obtained by Rössle (no lytic action of normal serum on paramecia) is probably due to the fact that he neglected the suitable concentration of electrolytes in the serum dilution.

IMMUNIZATION AGAINST PARAMECIA

Material for Immunization: A 10 days' culture of paramecia, containing large numbers, was subjected to an electric current to collect them and, at the same time, to get rid of as many of the contained bacteria as possible. A glass tube, 0.5 inch in diameter and of U form, both perpendicular parts of it having about the same length as the transverse part (= about 5 inches), was held firmly by a buret stand, and filled with paramecia culture, and a weak current was passed from one end of the tube to the other. I used 6 dry cells, each of which is of 1.5 volt, arranged by wire with an ammeter; the positive and negative poles were suspended in the liquid culture at the end of the tube. Under the above mentioned potential difference, I noticed 1/10 milliampere of current passing through the circuit. As soon as the circuit was closed the paramecia in the tube went over en masse to the cathode pole, with measurable speed. At first some paramecia remained around the anode pole or near the liquid surface, but after a short time all paramecia went over to the cathode. Some of them discharged trichocysts at the anode, that is, at the posterior end, and later were deprived of their motility and precipitate.

TABLE 17

RESULTS OF TESTS TO DETERMINE THE ACTION OF IMMUNIZED RABBIT SERUM ON PARAMECIA

	1	2	3	4	5	6	7
Rabbit serum 1, diluted with 0.6 % NaCl solution to 1 : 20....................	0.5	0.4	0.3	0.2	0.15	0.1	—
Action on paramecia in (1 hour in the incubator and then at room temperature):							
18 minutes............	+†	+†	+†	+++	+++	+++	+++
25 minutes............	†	†	†	+†	+++	+++	+++
45 minutes............	†	†	†	+†	+†	+†	+++
60 minutes............	†	†	†	+†	+†	+†	+++
20 hours...............	†	†	†	†	†	+†	+++
Microscopic examination at the end of 1 hour.........	D	D	D	D—			

If the cathode pole is held somewhat deep in the liquid, so that the paramecia pass through the cathode pole and gather near the surface of the liquid of that end in a large aggregation, most of the paramecia can be saved from the danger of injury by the current and many of them can be collected in a smaller amount of liquid. Repeating this process several times the bacteria in paramecia culture are eliminated almost completely.

By means of a centrifuge almost all the bacteria can be eliminated.

To each 0.1 c c of paramecia mass taken from the bottom of the centrifuge tube was added 1 c c of 0.6% NaCl solution and the thick suspension of paramecia was used as antigen. Three rabbits were used for immunization against paramecia. The antigen was given intraperitoneally in all 3 rabbits, weighing about 2,200 gm. each, 2.5 c c on Jan. 29, 3 c c Feb. 4, and again Feb. 14.

Through the whole course of the immunization we did not observe any particular symptom which could be regarded as an accidental result of the paramecia injection.

Action of Immunized Rabbit Serum on Paramecia: Blood was taken from the vein 5 days after the second injection of paramecia suspension. Clear serum was titrated immediately after the centrifugalization.

Microscopic examination at the end of 1 hour: Tubes 1-3 show an entire disintegration of all animals (indicated by D in Table 18), while in Tube 4 there are some animals swollen, with very large vacuoles; others make very slow movements (D—).

The smallest amount of the serum capable of killing the paramecia is 0.15-0.1 c c of 1:20 dilution. In all three of the immune serums tested results almost alike were obtained.

The same immune serum lost its action entirely by heating at 56 C. for 20-25 minutes, just as in the case of normal serum. Standing in an ice-box for 5 days, the active immune rabbit serum diminishes its titer to some extent, namely, the titer limit becomes 0.20 c c-0.15 c c of 1:20 dilution, and is caused by a diminished amount of complement.

On Feb. 20 other blood specimens were taken and titrated just as before; the titer limit of all 3 immune rabbit serums was between 0.2 c c and 0.15 c c of its 1:40 dilution.

TABLE 18

RESULTS OF TESTS IN ESTIMATING THE AMBOCEPTOR IN IMMUNE SERUM

	1	2	3	4	5	6	7	8	9	10	11
Inactive immune rabbit serum 1, diluted to 1 : 50....	0.4	0.3	0.2	0.15							
To 1 : 100..............					0.3	0.2	0.15				
To 1 : 200..............								0.3	0.2	0.15	—
0.6 % NaCl solution..........	—	0.1	0.2	0.25	0.1	0.2	0.25	0.1	0.2	0.25	0.5
Normal guinea-pig serum, diluted with 0.6 % NaCl to 1 : 20..................	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	—
Suspension of paramecia in water.......................	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):											
1 hour.................	†	†	†	†	†	†	†	+†	+†	+	+++
20 hours................	†	†	†	†	†	†	†	†	†	+†	+++

Estimation of the Amboceptor in the Immune Serum: Blood specimens were taken from immunized rabbits 15 days after the third injection of paramecia. Clear serum was inactivated.

The titer limit of normal guinea-pig serum, which is to be used as complement source, is in this case between 0.2 and 0.15 c c of its 1:20 dilution; 0.10 c c of that dilution is certainly incapable of killing the number of paramecia within 24 hours.

According to the results at least 0.2 c c of 1:200 dilution of inactivated immune serum, containing amboceptor only, to cover the shortage on amboceptor (normal amboceptor) in guinea-pig serum for combining with complement present in 0.10 c c of 1:20 dilution of fresh normal guinea-pig serum, should be added, to enable it to kill the number of paramecia used.

By parallel estimation of normal amboceptor in normal rabbit serum using the method previously described, we find that we have to add at least 0.2 c c of 1:20 dilution of inactive normal rabbit serum to cover the deficiency of amboceptor in 0.1 c c of 1:20 dilution of guinea-pig serum.

The different data regarding the amount of inactivated serum (that is, 0.2 c c of 1:200 dilution of the immune serums and 0.2 c c of 1:20 dilution of the normal rabbit serums) necessary to enable the same amount of normal guinea-pig serum (0.1 c c of 1:20 dilution) to act completely (cytolysis) give us the relation with regard to the amount of amboceptor in the immune and in the normal serum.

All three immune serums were tested by this method and each titer (used here only in a limited sense of the word) of amboceptor is respectively: Rabbit 1, 0.2 c c of 1:200 dilution; Rabbit 2, 0.2 c c of 1:200 dilution, and Rabbit 3, 0.3 c c of 1:400 dilution.

The immune serums contain about 10 or 15 times as much amboceptor as normal serum.

From the statements under the section on reactivation, it is evident that the action of normal (and immune) serum is a more complicated one than the action of ordinary salt alone.

The death of paramecia under the influence of serum may not be caused by amboceptor and complement. Certainly the disintegration of paramecia can be effected, just as in the case of hemolysis, by many other chemical, physical and toxic agencies.[14] * Excess of hydrogen or hydroxyl ion is injurious for paramecia. Normal blood is alkaline in reaction to litmus, the alkalinity being due to some carbonate. When examined according to physico-chemical methods the blood is found to be neutral, that is, it does not contain an excess of hydroxyl ions. The death of paramecia by the action of serums cannot be interpreted as due to the alkalinity (neutrality) of the serum.

The morphologic and physiologic alterations of paramecia under the influence of normal and immune serum depression of the motility, discharge of trichocysts, swelling of the body and finally disintegration — are surely due to the serum. I observed experimental facts which are to be interpreted as purely serologic, not merely toxicologic, namely, that serum, when heated at 56 C. for about half an hour, loses its activity, and that this can be regained (reactivated) by addition of fresh normal guinea-pig serum (as complement source); I noticed some special rôle of electrolyte in the action of serum on paramecia. Furthermore, I observed that an excess of inactivated serum (ambo-

[14] Rarratt, W.: Ztschr. f. allg. Physiol., 1904, 8, p. 438. Ibid., 1905, 5, p. 73. Bokorny, T.. Arch. f. Zellforschung, 1911, 7, p. 1. Neuhaus, H.: Arch. internat. d. pharmacol., 1910, 20, p. 393.

* As to the action of some bacterial toxins on paramecia I tried diphtheria and tetanus toxin and some culture filtrates of some other bacteria. Strong diphtheria and tetanus toxins have no effect on paramecia, even in 1:2 dilution. Culture filtrates of hemolytic staphylococcus and streptococcus (both 10 days cultures) also have no effect on paramecia. Cultures of B. pyocyaneus cause death of paramecia, but this seems to be due to the alkalinity of the filtrate, and not to any specific "pyocyanolysin"—an assumption which will be very easily proved by neutralization of the filtrate with N/20 HCl; the effect of the filtrate on paramecia is lost entirely when the filtrate is just completely neutralized. (See also E. O. Jordan, Jour. Med. Research, 1903, 10, p. 51.)

ceptor) mixed with an adequate amount of fresh normal guinea-pig serum (complement source) is rather unfavorable for the action of serum on paramecia — a phenomenon which is easily regarded as analogous to the so-called "deviation of complement" according to Neisser and Wechsberg.

The serum of the same species is, as a rule, most favorable for the complementation of the amboceptor (Ehrlich). H. Noguchi[15] reports that the immune hemolytic amboceptor (painted turtle immunized with the blood of Rana catesbiana) is entirely complemented by its own serum, slightly by the normal complement of speckled turtle, and not at all by the normal complement of bull-frog. The reactivation test of the inactive turtle and frog serums by normal guinea-pig complement, and of the former by the normal frog complement, illustrate this fact. We need not be discouraged, therefore, by the negative complementation in this case. Obviously, the complements in the normal turtle and frog serums do play an important part in causing death of infusoria by the action of these normal serums.

Tho I did not succeed in producing a highly potent antiserum against paramecia, the results support my opinion that the action of serum on paramecia is an entirely serologic, a beautiful example of cytolytic action of serum — a fundamentally different conception from that stated by Rössle. He says that phenomena which can be considered as analogous to bacteriolysis were not found at all, even when a low dilution of undiluted serum was used.

The failure of Rössle to recognize these phenomena which ultimately ought to be regarded as analogous to cytolysis may probably be due to the fact that he did not pay special attention to the significance of the electrolyte in the action of serum on paramecia.

RÉSUMÉ

Clear serum was diluted and distributed in a series of tubes to determine the smallest amount necessary to kill the same number of infusoria (Paramaecium caudatum).

Special attention was paid to the kind of salt and its concentration for the dilution of serum. Throughout the experiment 0.6% NaCl solution was used, unless special note is made.

The following kinds of serums were tested: human, horse, sheep, hog, beef, guinea-pig, rabbit, pigeon and frog and turtle.

[15] Bull. Univ. of Penn., 1902, 15, p. 301.

The toxic action of serum consists in depression of motility, discharge of trichocysts, swelling and finally disintegration of the bodies of paramecia. Colpoda is somewhat more resistant than Paramaecium.

Acting substance or substances can be adsorbed by various adsorbents.

The toxic activity disappears when serum is heated at 56 C. for about half an hour (warm-blooded animals) or at 53 C. for 20 minutes (cold-blooded animals). The reactivation was tested with fresh normal guinea-pig serum as complement source. Fresh guinea-pig serum contains relatively more complement than is required to combine with its own amboceptor.

A phenomenon similar to the so-called "complement deviation" was observed.

Evidently the action of serum on paramecia is analogous to bacteriolysis and hemolysis. Inactive serum of cold-blooded animals (frog and turtle), however, cannot be complemented by the normal complement either of guinea-pig serum or of frog serum.

Immune rabbit serums contain about 10-15 times as much amboceptor as the normal rabbit serum.

The normal serums of cold-blooded animals have a very much stronger activity than the serum of warm-blooded animals.

The morphologic and physiologic alterations of paramecia observed under the influence of normal and immune serums lead us to conclude that the action of serum on infusoria is typically serologic—a beautiful example of the mechanism and nature of cytolysis. This is a fundamentally different conception from that of Rössle.

LYSIS AND AGGLUTINATION OF RED CORPUSCLES OF WARM-BLOODED ANIMALS BY THE NORMAL SERUM OF COLD-BLOODED ANIMALS

M. Takenouchi

From the Department of Hygiene and Bacteriology, University of Chicago

INTRODUCTION

In 1902 H. Noguchi[1] made extensive observations on the agglutination and solution of red corpuscles by the serum of different cold-blooded animals. He states that the serum of painted turtles have no lytic activity for the blood corpuscles of flat fish, congo eel, common sea eel, speckled turtle, and snapping turtle; slight activity for the blood corpuscles of bull-frog, and tautog; complete lytic activity for the blood of dog-fish; while agglutinins for many kinds of blood corpuscles are present in large quantities.

Since then, however, there have been relatively few reports on this problem (E. Friedberger and A. Seeling (Rana esculenta var ridibunda),[2] K. Landsteiner and Hans Rock (frog serum),[3] Liefmann,[4] Fränkel,[4a] and especially K. Mazzetti[4b]). Mazzetti tested serum of different species of reptiles and amphibians against the red corpuscles of calf. He divided the animals tested into 3 classes: (1) animals whose serum have no hemolytic activity—lizard and turtle; (2) animals whose serum have slight hemolytic power—frog and green lizard, and (3) animals whose serum exercise strong hemolysis—salamander, toad and snakes in general.

Testing the lytic action with red corpuscles of a few other warm-blooded animals, besides the cold-blooded animals mentioned, he found that the activity of lytic serum is more or less general against the red corpuscles of warm-blooded animals. He states that 0.1 c c of fresh normal serum of turtle, the specific name of which he does not give, has no lytic action on 1 c c of 5% suspension of red corpuscles of turtle, lizard, frog, toad, snake, guinea-pig, rabbit and calf.

[1] Bull. Univ. of Penn., 1902, 14, p. 438.
[2] Centralbl. f. Bakt., O., I, 1908, 46, p. 421.
[3] Ztschr. f. Immunitätsforsch., 1912, 14, p. 14.
[4] Berl. klin. Wchnschr., 37, 1900, p. 1682.
[4a] Ztschr. f. Immunitätsforsch., 1911, 10, p. 415.
[4b] Ibid., 1913, 18, p. 132.

In the course of my study[5] of the action of different serums on paramecia I observed the occurrence of lysins in the serum of the painted turtle (Chrysemys picta) against the blood of rabbit and few other warm-blooded animals. This fact seems to be in contradiction to the statement of Mazzetti.

In this paper the hemolysins and hemagglutinins of the turtle serum will be discussed.

TABLE 1

TESTS TO DETERMINE LYSIS AND AGGLUTINATION OF TURTLE SERUM

	1	2	3	4	5	6	7
Dilution of the turtle serum with 0.85 % NaCl solution to 1 : 5............	0.5	0.4	0.3	0.2	0.15	0.1	—
0.85 % NaCl solution.........	—	0.1	0.2	0.3	0.35	0.4	0.5
5 % suspension of red corpuscles of different kinds of animals in 0.85 % NaCl solution....................	0.5	0.5	0.5	0.5	0.5	0.5	0.5
After 3 hours at room temperature:							
Human—							
Lysis....................	+++	+++	+++	+	±	—	—
Agglutination...........	+++	+++	+++	+++	—	—	—
Hog—							
Lysis....................	+++	+++	+++	+++	+++	+	—
Agglutination...........	+++	+++	+++	+++	+++	+++	—
Calf—							
Lysis....................	+++	++	+	—	—	—	—
Agglutination...........	+++	+++	+++	+++	—	—	—
Guinea-pig—							
Lysis....................	—	—	—	—	—	—	—
Agglutination...........	+++	+++	+++	+++	—	—	—
Rabbit—							
Lysis....................	+++	+++	+++	+++	+++	+++	—
Agglutination...........	+++	+++	+++	+++	+++	+++	—
Pigeon—							
Lysis....................	—	—	—	—	—	—	—
Agglutination...........	+++	+++	++	—	—	—	—
Sheep—							
Lysis....................	++	+	—	—	—	—	—
Agglutination...........	+++	++	—	—	—	—	—

NORMAL HEMOLYSIS AND HEMAGGLUTININS IN THE SERUM OF THE PAINTED TURTLE (CHRYSEMYS PICTA) AND OTHER SPECIES

All turtles were obtained in the beginning of March and kept in a box with some tap water at room temperature of the animal house of this laboratory. They were therefore in the condition of their hibernation when they were bled.

The turtle serum is said to have no, or at least not an appreciable amount of, hemolysins against different kinds of red corpuscles of cold-blooded animals (Noguchi, Mazzetti), as well as against the corpuscles of guinea-pig, rabbit and calf (Mazzetti).

Testing the normal serum of Chrysemys picta, I found that this serum contains an appreciable amount of hemolysins for the red corpuscles of different warm-blooded animals, besides normal agglutinins against different kinds of

[5] Takenouchi: Jour. Infect. Dis., 1918, 23, p. 396.

red corpuscles of cold-blooded animals. I tested the red corpuscles of the following animals to determine their reaction to agglutination and dissolution by normal turtle serum.

Human: positive lysis and agglutination.
Calf: positive lysis and agglutination.
Sheep: slightly positive lysis and agglutination.
Hog: strong positive lysis and agglutination.
Rabbit: strong positive lysis and agglutination.
Guinea-pig: negative lysis and positive agglutination.
Pigeon; no lysis and positive agglutination.

As is usual when biologic laking agents act on the red corpuscles of different kinds of animals, the hemolytic agent of the normal turtle serum, even in quantities more than sufficient to liberate all the hemoglobin, does not destroy the structure of the erythrocytes. Agglutination takes place, in almost all cases, quicker than the lysis; hemoglobin is generally liberated from the red corpuscles which are already agglutinated in more or less marked degree.

TABLE 2

SUMMARY OF RESULTS OF TESTS WITH DIFFERENT SPECIES OF TURTLES

	1 Chrysemys picta	2 Chrysemys belli	3 Chrysemys elegans	4 Melaco-clemmys lesueurii	5 Melaco-clemmys (geographica?)
Human—					
Lysis	0.2				
Agglutination	0.2				
Hog—					
Lysis	0.1	0.2	0.15	0.2	0.3
Agglutination	0.1	0.15	0.15	0.1	0.4
Calf—					
Lysis	0.4	—	0.5	—	—
Agglutination	0.2	0.3	0.15	0.4	—
Guinea-pig—					
Lysis	—	0.3	0.15	0.4	0.4
Agglutination	0.2	0.2	0.15	0.15	0.4
Rabbit—					
Lysis	0.1	0.1	0.1	0.15	0.2
Agglutination	0.1	0.1	0.1	0.1	0.3
Pigeon—					
Lysis	—	—	—	—	—
Agglutination	0.3	0.3	0.1	0.3	—

After the complete lysis has taken place, the ghosts are also agglutinated into many flakes or into a compact mass or have the appearance of a membrane floating in the hemoglobin solution. Using the terminology of Stewart,[6] the process of hemolysis by the turtle serum is really hemochromolysis and not hemocytolysis.

Several other different species of turtles, Chrysemys belli, Chrysemys elegans, Melacoclemmys lesueurii, and Melacoclemmys (geographica?), were tested with the same method on hemolysins and hemagglutinins, and the results summarized in Table 2. The titer limits of hemolysis and agglutination with different kinds of blood corpuscles are given in amount of serum dilution of 1:5.

The serum of different species of normal turtle contains different amounts of hemolysins and hemagglutinins for corpuscles of different kinds of animals.

[6] Jour. Pharmacol. and Exper. Therap., 1909, I, p. 89.

INFLUENCE OF LOW TEMPERATURE ON THE HEMOLYTIC AND HEMAGGLUTINATING PROCESS BY THE NORMAL TURTLE SERUM

It is well known that temperature plays an important part in the development of hemolysis. At a temperature approaching freezing it is usually absent in serum of warm-blooded animals. Agglutination, on the other hand, is not prevented by low temperature (Flexner[7]). Friedberger and Seeling[8] observed that the serum of the frog, unlike the serum of warm-blooded animals, causes complete hemolysis at 0 C., tho at a much slower rate.

The relation between the temperature and the readiness with which the lysis and agglutination of red corpuscles of rabbit blood succumb to the effects of turtle serum, will be seen in the following experiment.

TABLE 3

Effect of Temperature on Lysis and Agglutination

	1	2	3	4	5	6	7
Serum of Chrysemys picta diluted with 0.85 % NaCl to 1 : 10	0.5	0.4	0.3	0.2	0.15	0.1	—
0.85 % NaCl solution	—	0.1	0.2	0.3	0.35	0.4	0.5
5 % suspension of rabbit corpuscles	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lysis at 23 - 24 C.:							
15 minutes	+++	+++	+++	—	—	—	—
20 minutes	+++	+++	+++	++	+	—	—
60 minutes	+++	+++	+++	+++	+++	±	—
Lysis at 7 C.:							
2 hours	+++	—	—	—	—	—	—
3 hours	+++	+++	—	—	—	—	—
Lysis at 1 - 5 C.:							
2 hours	+*	—*	—*	—*	—*	—	—
3 hours	+++	++	—	—	—	—	—

* The agglutination of the corpuscles takes place in 1 hour in the first 5 tubes of the last series (at a temperature of 1 - 1.5 C.) in more or less marked degree.

Testing with 1 : 5 dilution of the serum of Chrysemys picta, we get the following result:

TABLE 4

Effect of Temperature when a Different Dilution is Used

	1	2	3	4	5
Turtle Serum 1 : 5	0.5	0.4	0.3	0.2	—
0.85 % NaCl	—	0.1	0.2	0.3	0.5
5 % suspension of rabbit corpuscles	0.5	0.5	0.5	0.5	0.5
Temperature: Time:					
At 15-17 C. 3 minutes, lysis	+++	+++	+++	++	—
8-8.5 C. 15 minutes, lysis	±	—	—	—	—
20 minutes, lysis	+++	-	—	—	—
0-1.0 C. 27 minutes, lysis	+	—	—	—	—
40 minutes, lysis	+++	++	+	—	—

[7] Bull. Univ. of Penn., 1902, 15, p. 324.

[8] Centralbl. f. Bakteriol., O., 1, 1908, 46, p. 421.

ACTION OF TURTLE SERUM ON HARDENED CORPUSCLES

It is stated[9] that red corpuscles which have been hardened in Hayem's fluid are still lysable by lytic serums. Noguchi[10] tested the action of agaricin, saponin, and tetanolysis on the red corpuscles of man, guinea-pig, and rabbit, hardened in Hayem's solution, formalin, and alcohol-ether, but did not observe any solution of the red cells or liberation of hemoglobin. On the other hand, corpuscles hardened in Hayem's solution and formalin are still capable of agglutination with ricin and the venom of cobra, moccasin, copperhead and rattlesnakes as well as with normal horse serum. Von Dungern and Coca[11] announced that osmic-hardened corpuscles can be laked by foreign serum. According to Stewart, sublimate fixed corpuscles at a certain stage of fixation

TABLE 5

TESTS TO DETERMINE THE ACTION OF TURTLE SERUM ON HARDENED CORPUSCLES

		1	2	3	4	5	6	7
Active normal serum of Chrysemys picta, diluted to 1:5 with 0.85 % NaCl solution		0.5	0.4	0.3	0.2	0.15	0.1	—
0.85 % NaCl solution		—	0.1	0.2	0.3	0.35	0.4	0.5
5 % suspension of hardened rabbit corpuscles in 0.85 % NaCl solution		0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of Fixation in Hayem's Solution:	Time of Observation:							
One hour	15 mins.							
	Lysis	—	—	—	—	—	—	—
	Agglutination	++	++	+	+	—	—	—
	40 mins.							
	Lysis	—	—	—	—	—	—	—
	Agglutination	++	++	++	+	+	—	—
	1½ hr.							
	Lysis	+	+	±	—	—	—	—
	Agglutination	++	++	++	+	+	—	—
	20 hr.							
	Lysis	+	+	+	—	—	—	—
	Agglutination	++	++	++	++	+	+	—
Two hours	15 mins.							
	Lysis	—	—	—	—	—	—	—
	Agglutination	++	++	+	+	—	—	—
	1 hr.							
	Lysis	—	—	—	—	—	—	—
	Agglutination	++	++	++	+	+	—	—
	20 hr.							
	Lysis	—	—	—	—	—	—	—
	Agglutination	++	++	++	+	+	+	—
Three hours*								

* No hemolysis took place in any tube; the degree of agglutination is just the same as above (2 hours' fixation).

do not lake in distilled water at room temperature, but lake in ammoniacal water, and are also laked in distilled water on heating. In blood partially fixed by formaldehyd and laked by saponin the ghosts are not broken up by water, as in ordinary blood laked by saponin.[8]

It was found that the hardened corpuscles of rabbit blood cannot be dissolved by the normal turtle serum (Chrysemys picta), but are agglutinated just as readily as the original blood corpuscles.

[9] Matthes: München. med. Wchnschr., 1902, 49, p. 8.

[10] Bull. Univ. of Penn., 1902, 15, p. 327.

[11] Berl. klin. Wchnschr., 1907, 44, p. 1471.

Blood, taken from a normal rabbit directly into the Hayem's solution, was placed at room temperature for different lengths of time until the corpuscles were centrifugalized repeatedly and washed thoroughly with physiologic salt solution.

The solution of corpuscles, or rather the liberation of hemoglobin, which took place in the first series was due to insufficient fixation.

CONCENTRATION OF ELECTROLYTES AND AGGLUTINATION OF HARDENED CORPUSCLES

In the action of the agglutinin on hardened corpuscles of rabbit blood I noticed that a certain salt concentration is invariably necessary for the agglutination of hardened corpuscles—a fact which agrees with that of the agglutination of different bacteria by specific or normal agglutinins.

TABLE 6

SALT CONCENTRATION NECESSARY IN THE ACTION OF AGGLUTININ ON HARDENED CORPUSCLES

	1	2	3	4	5	6
Turtle serum, diluted with 0.85 % NaCl solution to 1 : 5	0.3	0.3	0.3	0.3	0.3	0.3
Distilled water	—	0.1	0.2	0.3	0.4	0.5
0.85 % NaCl solution	0.5	0.4	0.3	0.2	0.1	—
10 % suspension of hardened rabbit corpuscles in water	0.2	0.2	0.2	0.2	0.2	0.2
Time of Observation:						
15–18 minutes Agglutination	+++	+++	++	+	+	—
30 minutes Agglutination	+++	+++	+++	++	+	—
20 hours Agglutination	+++	+++	+++	+++	++	—

THE INACTIVATION OF NORMAL SERUM OF CHRYSEMYS PICTA

The heat lability of normal hemolytic complement in the serum of Chrysemys picta was determined. The serum was heated for 30 minutes to a temperature varying from 45-60 C., after which the lysis was again tested. Heating at 50 C. for 30 minutes is sufficient for complete inactivation of complement in the serum of Chrysemys picta, while heating at 45 C. for 30 minutes left the complement almost intact.

THE INACTIVATION OF TURTLE SERUM BY DIGESTION WITH DIMINISHED SALT CONCENTRATION (SACHS AND TERUUCHI)[12]

The digestion method was used with active fresh as well as somewhat old serums of the turtle, and the results were similar to those observed by Sachs and Teruuchi in their study with the serums of warm-blooded animals, namely, (1) the normal active turtle serum, which has been kept over 48 hours in an ice-box, can be inactivated when it is digested with water in a dilution of 1:9, but not of 1:5 (the dilution of the serums with water to 1:9-10 is said to be most favorable for inactivation); (2) fresh turtle serum cannot be inactivated by this method at all.

[12] Berl. klin. Wchnschr., 1907, 16, p. 467; 17, p. 520; 19, p. 602; and Tsuda, Ibid., 1908, 8, p. 399.

REACTIVATION OF INACTIVE TURTLE SERUM

Reactivation of inactivated normal turtle serum was attempted many times with normal complement of guinea-pig and of frog, but no reactivation was observed in either case.

HEAT LABILITY OF HEMAGGLUTININS IN THE SERUM OF CHRYSEMYS PICTA

Noguchi[13] showed that the agglutinins in the serum of Limulus polyphemus have varying degrees of heat lability, altho temperatures of 40 C., when continued for 30 minutes, diminish the activity of all the agglutinins. Temperatures approaching 65 C. seem to destroy wholly the agglutinating power of the serum for erythrocytes.

The results of my experiments with the inactive serum of Chrysemys picta and different kinds of red corpuscles of warm-blooded animals are as follows:

(*a*) The agglutinins for hog, rabbit and pigeon corpuscles do not show any marked diminution of activity on heating at 50 C. for 30 minutes or at 53-54 C. for 15 minutes. Agglutinin for rabbit corpuscles in the normal turtle serum is not entirely destroyed by heating the serum even at 60 C. for 30 minutes, when the serum becomes almost coagulated.

(*b*) The agglutinins for calf and sheep corpuscles seem to be entirely destroyed by heating at 50 C. for 30 minutes. The agglutinin for guinea-pig corpuscles in the turtle serum is destroyed to a great extent by heating the serum at 53 C. for 15 minutes.

The highest temperature at which the agglutinins in the normal turtle serum could be completely destroyed was not determined. In this experiment the agglutinins in the serum of Chrysemys picta are multiple in their nature; in other words, the agglutinins for some kinds of red corpuscles of warm-blooded animals are different from those for other corpuscles. The question regarding the multiplicity of the serum agglutinins of cold-blooded animals is answered by Noguchi in the affirmative. According to the result of my experiment on absorption the conclusion that the serum of Chrysemys picta contains several kinds of agglutinins seems most likely to be correct.

ABSORPTION TESTS

Fresh normal active serum of Chrysemys picta was diluted to 1:5 with 0.85% salt solution; to each 10 c c of this dilution 0.7 c c of red corpuscles of different kinds of animals is added. Each tube is placed in ice water at a temperature between 0 C.-1.5 C. for different lengths of time (from 10-17 minutes) and then centrifugalized. The supernatant fluid of each serum dilution and blood corpuscles is tested with a different kind of corpuscle for agglutination and lysis.

To avoid hemolysis during the absorption, the time of absorption must be controlled carefully, and the temperature must be kept low enough to make the rate of absorption of complement as slow as possible. The method of inactivating the serum by heating should not be employed here because normal hemagglutinins for some kinds of corpuscles are very heat labile.

[13] Centralbl. f. Bakteriol., 1903, O., I, 34, p. 286.

(*a*) Absorption of turtle serum (Chrysemys picta) by the red corpuscles of human blood.

Turtle serum (1:5) absorbed by the red corpuscles of human blood for 7 minutes at 0-1.5 C.; 3 minutes for centrifugalization.

TABLE 7*

SHOWING THE ABSORPTION OF TURTLE SERUM BY RED CORPUSCLES OF HUMAN BLOOD

Supernatant	0.5	0.4	0.3	0.2	0.1	—
0.85 % NaCl	—	0.1	0.2	0.3	0.4	0.5
5 % suspension of corpuscles	0.5	0.5	0.5	0.5	0.5	0.5
a. Human—						
Lysis	±	—	—	—	—	—
Agglutination	+	—	—	—	—	—
b. Calf—						
Lysis	—	—	—	—	—	—
Agglutination	+++	+++	+++	—	—	—
c. Hog—						
Lysis	+++	+++	+++	+++	+	—
Agglutination	+++	+++	+++	+++	—	—
d. Rabbit—						
Lysis	+++	+++	+++	+++	+++	—
Agglutination	+++	+++	+++	+++	+++	—
e. Hardened Rabbit—						
Lysis	—	—	—	—	—	—
Agglutination	+++	+++	+++	+++	+++	—

* Many readings were made at different intervals of time, but only the one at the 6th hour will be given in Tables 7-11.

(*b*) Absorption by the red corpuscles of rabbit blood; 7 minutes for absorption and 3 minutes for centrifugalization.

TABLE 8

ABSORPTION TESTS USING RABBIT BLOOD

Supernatant	0.5	0.4	0.3	0.2	0.1	—
0.85 % NaCl	—	0.1	0.2	0.3	0.4	0.5
5 % suspension of corpuscles	0.5	0.5	0.5	0.5	0.5	0.5
a. Human—						
Lysis	++	—	—	—	—	—
Agglutination	+	—	—	—	—	—
b. Calf—						
Lysis	—	—	—	—	—	—
Agglutination	+	±	—	—	—	—
c. Hog—						
Lysis	++	+	—	—	—	—
Agglutination	++	—	—	—	—	—
d. Rabbit—						
Lysis	+++	+++	+++	+	—	—
Agglutination	+++	+++	+++	+++	+	—
e. Hardened Rabbit—						
Lysis	—	—	—	—	—	—
Agglutination	+++	+++	+++	+++	—	—

From the results obtained in the absorption experiments described, the following statements seem warranted, though the time for the absorption was not sufficient in some cases because it was necessary to avoid any hemolysis which might occur during the absorption.

The result is somewhat different from that which was obtained by

(*c*) Absorption by the red corpuscles of hog blood; 7 minutes for absorption, 3 minutes for centrifugalization.

TABLE 9

ABSORPTION TESTS USING HOG BLOOD

Supernatant	0.5	0.4	0.3	0.2	0.1	—
0.85 % NaCl	—	0.1	0.2	0.3	0.4	0.5
5 % suspension of corpuscles	0.5	0.5	0.5	0.5	0.5	0.5
a. Human—						
Lysis	++	—	—	—	—	—
Agglutination	++	+	—	—	—	—
b. Calf—						
Lysis	—	—	—	—	—	—
Agglutination	+	+	—	—	—	—
c. Hog—						
Lysis	—	—	—	—	—	—
Agglutination	+++	++	+	—	—	—
d. Rabbit—						
Lysis	+++	+++	+++	+++	++	—
Agglutination	+++	+++	+++	+++	+	—
e. Hardened Rabbit—						
Lysis	—	—	—	—	—	—
Agglutination	+++	+++	+++	+++	+++	—

(*d*) Absorption by the red corpuscles of calf blood for 17 minutes and centrifugalization for 3 minutes.

TABLE 10

ABSORPTION TESTS USING CALF BLOOD

Supernatant	0.5	0.4	0.3	0.2	0.1	—
0.85 % NaCl	—	0.1	0.2	0.3	0.4	0.5
5 % suspension of corpuscles	0.5	0.5	0.5	0.5	0.5	0.5
a. Human—						
Lysis	+	—	—	—	—	—
Agglutination	+	—	—	—	—	—
b. Calf—						
Lysis	—	—	—	—	—	—
Agglutination	+	—	—	—	—	—
c. Hog—						
Lysis	+++	++	+	+	—	—
Agglutination	++	++	+	+	—	—
d. Rabbit—						
Lysis	+++	+++	+++	+++	—	—
Agglutination	+++	+++	+++	+++	+++	—
e. Hardened Rabbit—						
Lysis	—	—	—	—	—	—
Agglutination	+++	+++	+++	+++	—	—

Noguchi in his study of hemagglutinins in the serum of Limulus polyphemus against the different kinds of corpuscles of cold-blooded animals, where the absorption of the agglutinin for one species left those for others in practically undiminished quantities.

The lysins and agglutinins for hog corpuscles can be absorbed by the red corpuscles, not only of hog blood, but also of rabbit and of calf blood, tho not by human blood corpuscles.

(*e*) Absorption by hardened rabbit corpuscles for 20 minutes; 3 minutes for centrifugalization.

TABLE 11

SHOWING THE ABSORPTION BY HARDENED RABBIT CORPUSCLES

Supernatant	0.5	0.4	0.3	0.2	0.1	—
0.85 % NaCl	—	0.1	0.2	0.3	0.4	0.5
5 % suspension of corpuscles	0.5	0.5	0.5	0.5	0.5	0.5
a. Human—						
Lysis	+++	++	++	+	—	—
Agglutination	+++	+	+	—	—	—
b. Calf—						
Lysis	+++	+++	++	—	—	—
Agglutination	+++	+	±	—	—	—
c. Hog—						
Lysis	+++	+++	+++	++	+	—
Agglutination	+++	+++	++	—	—	—
d. Rabbit—						
Lysis	+++	+++	+++	+++	+++	—
Agglutination	+++	+++	+++	+++	++	—
e. Hardened Rabbit—						
Lysis	—	—	—	—	—	—
Agglutination	+++	++	+	—	—	—

The lysins and agglutinins for rabbit corpuscles and the agglutinins for hardened corpuscles of rabbit blood are absorbed most strongly by the corpuscles of the same animals and cannot be absorbed very much by other kinds of corpuscles.

The lysins and agglutinins for human corpuscles are almost equally absorbed by human, rabbit, calf and hog corpuscles.

The lysins for calf blood corpuscles can be absorbed by different kinds of corpuscles.

The hardened corpuscles of rabbit blood cannot absorb very much any kind of lysins in the turtle serum.

RÉSUMÉ

The normal serum of different species of turtle contain different amounts of lysins and agglutinins for different kinds of corpuscles of warm-blooded animals.

The normal serum of turtle (Chrysemys picta) contains several lysins and agglutinins for erythrocytes of warm-blooded animals.

The normal lysins of the turtle serum lake the red corpuscles of warm-blooded animals even at freezing temperature.

The red corpuscles of rabbit blood hardened by Hayem's solution cannot be laked by the turtle serum, unless the fixation is incomplete.

For the agglutination of hardened corpuscles a certain concentration of electrolyte is necessary.

Reactivation of inactive turtle serum by normal complement of guinea-pig or frog was tested with negative result.

The agglutinins of turtle serum for erythrocytes of warm-blooded animals have varying degrees of heat lability.

Red corpuscles of some species of the tested animals can absorb agglutinins and lysins for erythrocytes of other animals. The specificity of normal agglutinins and lysins in turtle serum (Chrysemys picta) for erythrocytes of warm-blooded animals seems not to be so strict as claimed by Noguchi in the serum of Limulus polyphemus and Musterus canis for erythrocytes of different cold-blooded animals.

However, the serum of turtle (Chrysemys picta) seems to contain not only a multiplicity of agglutinins, but also a multiplicity of lysins for red corpuscles of warm-blooded animals.

THE USE OF GOLD SALTS IN THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS IN GUINEA-PIGS

XVIII. STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS

Lydia M. DeWitt

From the Otho S. A. Sprague Memorial Institute and the Department of Pathology, University of Chicago

The use of gold in therapy is by no means new and a complete survey of the history and literature has been impracticable. The earliest reference which I have found in the literature states that Abu Moussa Djafar or Geber, surnamed the Wise, who was born at Houran in Mesopotamia in the middle of the 8th century, recommended the use of gold as a cure for every disease of men, animals and plants, asserting that all the metals were diseased except gold.

Sprengel's Arzneikunde (1800) in the chapter on Medical Culture among the Arabians, refers to the many medical and philosophical treatises of Ebn Sina (better known as Avicenna) who lived from 978-1036 A. D. This ancient philosopher recommended gold, silver and other metals for internal use as blood purifiers. Gold foil was also used in his time to coat pills in the belief that the gold coating increased their efficacy.

Naturally the alchemists in their eager interest in gold did not neglect its use in medicine and Paracelsus (1493-1541), the alchemist and chemist who insisted that the true use of chemistry was to make medicines rather than gold, considered gold as a specific in all affections of the heart; he based this belief on the fact that in the mystic scale, gold is in harmony with that viscus. The Paracelsian "Elixir of Life" or panacea for all ills was said to be composed of gold and mercury. The so-called potable gold or liquid gold and its use in medicine dates from this same period and was highly recommended by Roger Bacon and many others. The Sceptical Chymist (Boyle) criticises its use but admits some wonderful cures. In 1540, Lecoque is said to have cured syphilis by the use of gold, which was much like mercury in its action. Hahnemann ascribed great curative power to gold and Burnett recommended trituration of gold leaf 1 : 1,000,000 as a heart tonic.

The use of gold as a cure for alcoholism was long exploited under the name of the Keeley Gold Cure but in 1891 it was confessed that the remedy used contained no gold and that the name was used with fraudulent intent.

In 1917, Marsalongo and Vivaldi reported 32 cases of typhoid and paratyphoid fever successfully treated with colloidal gold. At different times and by different workers, gold has been used in the treatment of syphilis, heart disease, cancer, chronic Bright's disease, typhoid fever, and chronic rheumatism.

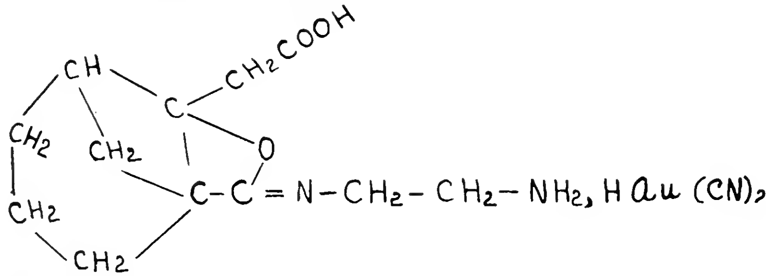
Received for publication May 20, 1918.

It is also said to have a special influence on the lithemic and fatty degenerations, and on various spinal and cerebral scleroses.

The use of gold in the treatment of tuberculosis, according to White, dates from the time of Paracelsus; for many years after his time, gold in combination with mercury was depended on in the treatment of the cachexias resulting from phthisis, scrofula, syphilis, etc. After a time, however, some serious accidents led to a discontinuance of its use until 1810 when Chrestien brought it back into prominence. In 1890, Robert Koch stated that gold cyanid completely inhibits the growth of tubercle bacilli in dilution of 1:2,000,000 but that it is entirely without influence in the animal body. In 1891, J. B. White recommended the use of double chlorid of sodium and gold combined with iodid of manganese in the treatment of tuberculosis. The effects noted were a distinct prolongation of life in animals, and in human patients gain in weight and appetite, diminution of cough and sputum and râles, absorption of abnormal deposits, and improvement of blood conditions. Merrill also used White's treatment on 20 patients for 2 years and considers its value unquestionable. In 1891 also, the well known Gibbes and Shurley treatment for tuberculosis was published. This consisted of a preliminary iodin treatment for 2 weeks, then double chlorid of gold and sodium for 10 days, then again the iodin, and so on, the 2 treatments alternating. During the time of the treatment, inhalation of chlorin gas was recommended. They reported about 50% of their cases "practically cured."

The next and the most extended group of experiments on the use of gold salts in tuberculosis were published between 1912 and 1915 by a number of German investigators. Several workers reported results from the use of gold potassium cyanid especially in tuberculosis of the skin. Meyer reports good results in general tuberculosis, especially if the gold salt is combined with some substance like borcholin which is said to dissolve the fatty sheath of the tubercle bacillus. Oberstadt reported good influence on the skin lesions, but no case of perfect recovery. He thinks the effect is produced more rapidly if tuberculin treatment is combined with the treatment with the gold salt. Bruck and Glück, after a series of rather unsuccessful experiments in guinea-pigs, used gold potassium cyanid in the treatment of lupus. They ascribed their failure in guinea-pigs to the fact that the gold salt needs to be injected intravenously at frequent intervals and frequent intravenous injections are impracticable in guinea-pigs. Bettmann obtained similar results in the same type of cases. Junker reports good effects from the use of gold salts in pulmonary as well as in skin tuberculosis in patients. Rosenthal injected gold tricyanid directly into the diseased area in men, claiming good results. Poor and Geber also report success from the use of gold potassium cyanid in patients having skin tuberculosis. A series of 7 or more papers, published by Spiess and Feldt, together or separately, between 1912 and 1916, form the most extensive contribution to the literature on the treatment of tuberculosis by means of different salts of gold. These authors consider gold cyanid and gold potassium cyanid too toxic for use in human patients. As cantharidin had earlier been found to have a marked affinity for tuberculous tissues, they conceived the idea of reducing the toxicity of the cantharidin and using it as a carrier for the specific gold cyanid. They were able to reduce the toxicity of the cantharidin 700 times without altering its affinity for tubercles by condensing it with ethylenediamin. This compound was quite nontoxic and combined

readily with gold cyanid. They used first the auric salts but found them 100-1,000 times less efficacious than the aurous salt and therefore in all their later work they used mono-cantharidyl-ethylenediamin-aurous-cyanid, the formula of which they give as follows:



The compound was first tested on about 100 animals during 1½ years and was then used on human patients. They claim that its effects are due partly to a direct inhibitory action on the tubercle bacilli and partly to an indirect action on the defensive agencies of the body. It stimulates connective tissue formation and destroys disease products by its oxidizing power. In experimental tuberculosis in animals, they claim that weight was increased and life was prolonged and tubercles were replaced by scar tissue, especially in rabbits. In guinea-pigs, the effects were not so marked, a fact which they attribute to the necessity of administering the drug subcutaneously. Long continued subcutaneous treatment causes necrosis, and the development of gold tolerance. This was shown in vitro, as, after 1½ months, the bacilli grew on mediums containing 1 part of gold cyanid to 400,000 parts of agar, whereas at first they would not develop in a dilution of 1 part in 2,000,000.

In contrast to the above favorable reports on the use of gold salts in tuberculosis, Koch in 1890 stated that the gold salts were without effect in animals. Perutz and Seppel, Walther, and Schoenfeld admitted that they obtained good results at first, especially in early cases, but stated that these results were transient and did not interfere with the progress of the disease. Blendberger, even in cases of lupus, was unable to obtain results at all comparable to those reported by other workers. Pekanovich, Hauck, and Pasini all call attention to the dangers from the use of gold cyanids, since they are, according to Hauck, severe blood poisons, reducing the number of both red and white cells and causing a severe hemolytic icterus. Pekanovich also reported severe hemorrhages after their use.

On the physiologic and pharmacologic action of gold salts in the animal body, much has been written and it will not be practicable to review more than a few of the most important communications having special reference to the use of gold in tuberculosis. Pereira's materia medica of 1839 states that "its effects are similar to those of mercury. Its salts are corrosive, but less so than mercuric chlorid. In small doses, they are stimulant, promoting the secretion of skin glands, salivary glands and kidneys. In doses of 0.1 gm. in man, they may cause fever, a reaction which Chrestien considered necessary for curative effect. Within proper dosage, no ill effects are produced." Heubner says that the rapidly fatal dose of sodium-gold chlorid is 15 mg. of the salt per kg. of body weight or 7 mg. of gold. If such a dose is given, the blood pressure falls rapidly until it reaches 0 and the animal dies in a few minutes of respiratory

stasis, all the organs showing hyperemia, with occasional ecchymoses and larger hemorrhages. All the veins and capillaries are greatly dilated and often ruptured while the arteries are strongly contracted. In other words, Heubner regards gold salts as vascular poisons. A more chronic intoxication is also described by Heubner. By injecting very slowly and thus avoiding a certain concentration in the blood, more than the acutely fatal dose can be introduced without causing immediate death. In these chronic intoxications, there is no indication of capillary poisoning and no macroscopic change in the organs. Microscopically, the kidney presents the usual picture of metallic poisoning—hyperemia, epithelial degeneration, fat cylinders in the tubules. The lungs show areas of hepatization, and the smaller bronchi are closed by hyaline masses. Many pigmented granular cells are seen. Heubner was unable to find gold microscopically in the organs. Richards, working in Heubner's laboratory, reported finding metallic gold in the nuclei of many organ cells, a report which Heubner failed to corroborate. By chemical analysis, however, Heubner found gold in the blood one half hour after intravenous injection, in the liver, spleen, lungs, and kidneys. Hauck states that gold cyanids cause a great diminution of the erythrocytes and leukocytes in the blood with a severe hemolytic icterus. Junker also notes a rapid lowering of the hemoglobin content of the blood. Bruck and Glück, on the other hand, found no effect on the blood from the use of gold cyanids. Schumacher states that the gold cyanids quickly break down in the body and form probably gold protein compounds which soon release colloidal gold. As the cyanid group is soon synthesized to the nontoxic sulphocyanid or rhodanid, Schumacher thinks that the effects in the body both toxic and therapeutic are due to the colloidal gold and not to the cyanid radicle. Spiess and Feldt and others who are using the gold cyanid in the treatment of tuberculosis admit that hyperemias and hemorrhages result from their use but insist that these are confined to the tuberculous areas, resembling the tuberculin reaction and leading to healing. Colloidal gold solutions have also been injected intravenously. Busquet states that colloidal gold raises the blood pressure and increases the force of the heart beats, diminishing their number, while soluble gold salts have a quite opposite effect, lowering the blood pressure, increasing the frequency of heart beats and diminishing their amplitude.

Koch found that gold in the form of cyanids had very little bactericidal effect on the tubercle bacillus but completely inhibited its growth in a dilution of 1:2,000,000. This statement was verified by Rosenthal and by Behring for other bacteria. Behring, however, found that this high inhibitory power in water solutions was reduced in blood serum to 1:20,000-30,000. Feldt demonstrated that colloidal gold had an inhibitory effect nearly equal to that of gold cyanid, completely inhibiting the growth of tubercle bacilli in a dilution of 1:1,000,000. He also found that gelatin mediums containing 1 part of colloidal gold in 6,000 parts of gelatin remained sterile though left open for 45 days, while control tubes containing no gold were covered in 3 days with a growth of air organisms.

My own experiments on the bactericidal and inhibitory power of gold salts on tubercle bacilli correspond entirely with the findings of

Koch and Behring. DeWitt and Sherman reported that 1 part in 1,000 of gold chlorid is required to kill tubercle bacilli while 1% solutions of gold tricyanid were not always able to kill all the organisms. As opposed to this low bactericidal power, I have found that 1 part in 2,000,000 is able to inhibit completely the growth of these organisms in the test tube. On the other hand, I have found a certain tolerance develop very slowly. After 4 months' growth on agar containing gold, the bacilli would grow very well on mediums containing 1 part of gold in the form of gold potassium cyanid in 2,000,000 and slightly on tubes containing 1 part in 1,000,000. This is not nearly so marked a tolerance as was described by Feldt who states that after 1½ months, he obtained a good growth on dilutions of 1 part of gold cyanid in 400,000 of the mediums. Shiga, on the other hand, asserts that in his experiments the tubercle bacilli developed no tolerance toward gold potassium cyanid. The question of acquired tolerance is one of considerable importance in the chemotherapy of a disease like tuberculosis in which treatment must be continued for long periods. According to our results in the in vitro experiments, however, the tolerance of the tubercle bacillus to gold develops so slowly as not to be a very important factor in the consideration of the treatment of tuberculosis with gold.

The stimulation of the growth of tubercle bacilli by solutions of gold too dilute to kill or inhibit is another important consideration in the use of gold in chemotherapy. We have frequently noticed in our culture tubes that the higher dilutions contained a more luxuriant growth than the control tubes. Takenaka reported that gold salts in broth dilution gave the following results:

1 : 100,000,000	caused stimulation at first.
1 : 10,000,000	caused no inhibition and no stimulation.
1 : 2,000,000	caused no inhibition and no stimulation.
1 : 1,000,000	caused no inhibition but often stimulation.
1 : 500,000	caused no inhibition.
1 : 100,000	caused marked inhibition.

The importance of the consideration of stimulation of growth by a gold concentration below that required to inhibit the growth may easily be appreciated, since it is difficult to keep the concentration of gold in the animal body constantly at or above the point required for inhibition.

My own experiments began Jan. 26, 1915. Eighteen guinea-pigs were inoculated subcutaneously with a strain of human tubercle bacilli of fairly low virulence, the untreated controls dying of generalized

tuberculosis in from 4-10 months. Ten days after inoculation, treatment with gold salts was begun, partly by subcutaneous injection and partly by feeding. Aug. 25, 1915, Sept. 14, 1915, Nov. 17, 1915 and Dec. 6, 1915, and Feb. 28, 1916, other sets were inoculated and treated with gold salts. In most cases, treatment was begun 24 hours after inoculation. In these experiments, some animals were treated with auric cyanid, some with gold potassium cyanid and some with aurous cyanid. All were fed daily and all received injections once or twice a week, sometimes intracardiac and sometimes subcutaneous or intramuscular. The subcutaneous injections often caused ulceration and necrosis of the surrounding tissues; the intramuscular injections of oil emulsions resulted in infiltration of the muscles from the slow absorption, with stiffness, lameness and paralysis. The intracardiac injections were not entirely without danger and a number of animals died immediately after the injection either from hemorrhage or from the toxic effects of the gold cyanids. In many cases, as many as 16 or 18 heart injections were given to the same animals.

Later, desiring to try the effect of the cantharidyl-ethylenediaminaurous-cyanid recommended by Spiess and Feldt, and being unable to procure any on account of the war, I had some made in my laboratory according to the directions given in the German patent. This preparation was used in the treatment of about 30 tuberculous guinea-pigs. At first it was introduced by intracardiac injection, but later, having found by chemical analysis that it was quickly and completely absorbed from the subcutaneous tissue, the less dangerous method of subcutaneous injection was used. This preparation is much less toxic than the simple cyanids and caused no necrosis in the skin and subcutaneous tissue. Its power of inhibition, however, was somewhat less as the highest dilution which completely inhibited growth of the tubercle bacillus in the test tube was 1 part of gold in the form of aurocantan to 250,000 parts of agar instead of 1 part of gold to 2,000,000 parts of agar.

Having determined that in 1 week from 40-50% of the amount of gold introduced was excreted through the urine and feces, injections were made once a week to keep the concentration in the organism high enough to equal the level necessary for inhibition of the growth of the tubercle bacillus. The dose used at first was usually 2 mg. This was soon increased to 4 mg. and later to 5, then 8, and then 10 mg. of the preparation, about 40% of which is gold. If we could believe that this gold is uniformly distributed throughout the body and retains in the

body an inhibitory power equal to that which it possesses in the test tube, these doses repeated at intervals of 1 week would constantly maintain a concentration sufficient to prevent the growth of any tubercle bacilli in the body. Table 1 will show that this hope was not realized. The total amount of gold administered and the length of life of the animals, as well as the extent of the disease are indicated in the table and are compared with the length of life and extent of the

TABLE 1

A SUMMARY OF THE EFFECT OF TREATMENT WITH GOLD SALTS ON THE PROGRESS OF TUBERCULOSIS IN GUINEA-PIGS

Animals Treated with Gold								
Gold Salt	Mg. Gold	Days of Life	Local Glands	General Glands	Extent of Disease in			Notes
					Liver	Spleen	Lungs	
$AuK(CN)_2$	54.40	64	—	—	++	+++	+++	
$AuK(CN)_2$	92.48	77	+++	—	+++	+++	+++	
$AuK(CN)_2$	92.48	77	+++	+++	+++	+++	+++	
$AuK(CN)_2$	261.80	115	+++	+++	+++	+++	+++	
$AuK(CN)_2$	184.96	120	+++	—	+++	+++	+++	Pericarditis
$AuK(CN)_2$	178.84	125	+++	+++	+++	+++	+++	
$AuK(CN)_2$	189.72	134	+++	+++	+	++	+	
$Au(CN)_3$	145.55	101	+	—	+++	+++	—	
$Au(CN)_3$	211.58	132	+++	+++	+++	+++	+++	
$Au(CN)_3$	209.45	135	+	+	+	+++	+	
$Au(CN)_3$	237.85	150	+	—	+	+++	+++	
$Au(CN)_3$	249.92	160	+++	—	+++	+++	+++	
$Au(CN)_3$	130.64	177	+++	+++	+++	+++	+++	
$Au(CN)_3$	309.56	188	+	—	+++	+++	+++	
$Au(CN)_3$	173,24	211	+++	+++	+++	+++	+++	
AuCN	35.20	44	+	—	—	+	+	
AuCN	35.20	49	+	+	—	+	—	
AuCN	97.68	197	+++	+++	+++	+++	+++	
AuCN	129.36	237	+++	+++	+	+++	+++	
Aurocantan	1.6	7	++	+	—	—	—	
Aurocantan	0.53	9	—	—	—	—	—	Hemorrhage
Aurocantan	2.4	17	—	—	—	—	—	Acute infec.
Aurocantan	9.6	37	+++	+++	+++	+++	+++	
Aurocantan	4.8	42	+	—	—	—	—	Hemorrhage
Aurocantan	13.6	51	+++	++	+++	++	+++	
Aurocantan	13.6	57	+++	+++	+++	+++	+++	
Aurocantan	14.4	60	—	—	—	+++	+++	Killed
Aurocantan	5.06	68	++	+++	—	+	—	Hemorrhage
Aurocantan	28.0	90	+++	+++	+++	+++	+++	
Aurocantan	16.8	107	+	—	—	+++	+++	
Aurocantan	8.5	115	+	+	+	+	—	
Aurocantan	12.26	153	+	+	—	—	—	
Aurocantan	31.6	160	+	+	—	—	—	Hemorrhage
Aurocantan	15.06	181	+	++	—	+++	++	
Aurocantan	11.46	184	+	+++	++	+++	+++	
Aurocantan	90.8	227	+	+	+	+	—	Killed
Aurocantan	90.8	228	++	+++	+++	+	—	Killed
Aurocantan	62.0	232	+	+	+	—	—	Killed
Aurocantan	21.86	236	++	+++	+	+	++	Pneumonia
Aurocantan	17.06	242	+++	+++	+++	+++	+++	Pneumonia
Aurocantan	17.06	247	++	+++	+++	+++	+++	Pneumonia

TABLE 1

A SUMMARY OF THE EFFECT OF TREATMENT WITH GOLD SALTS ON THE PROGRESS OF TUBERCULOSIS IN GUINEA-PIGS

Animals Not Gold Treated (Controls)

Days of Life	Local Glands	General Glands	Extent of Disease in Liver	Extent of Disease in Spleen	Extent of Disease in Lungs	Notes
57	+++	+++	+++	+++	+	Pericarditis; pneumonia
57	+++	+++	+++	+++	+	Pericarditis
62	+++	+++	+	++	+++	
92	+++	+++	+++	+++	+++	Pericarditis
97	+++	++	++	++	+	Hemorrhage
98	+++	+++	+++	+++	+++	
100	+++	+++	+++	+++	+++	
105	+++	+++	+++	+	++	Peritonitis
120	+++	—	+++	+++	+++	Pericarditis
122	+++	+	+	+	++	Killed
122	+	+	+++	+++	+++	
133	+++	+++	+++	+++	+++	
143	+++	+++	+++	+++	+++	
147	+++	+++	+++	+++	+++	
167	+++	+++	+++	+++	+++	
174	+++	+++	+++	+++	+++	Tuberculosis of omentum
176	+++	+++	+++	+++	+++	
180	+++	+++	+++	+++	+++	
181	+++	+++	+++	+++	+++	
184	+++	+++	+++	+++	+++	
197	+++	+++	+++	+++	+++	
238	+++	+++	+++	+++	+++	
243	++	—	+++	+++	+++	
264	+++	+++	+	+++	+++	
288	+++	+++	+++	+++	+++	
293	+++	+++	+++	+++	+++	
315	+++	+++	+++	+++	++	
27	+++	+++	—	+++	—	
45	—	—	—	+	+	
59	+++	+++	+++	+++	+++	
104	+++	+++	+++	+++	+++	
105	+++	+++	+++	+++	+++	
105	+	++	+++	+++	+++	
113	+	—	—	—	—	
142	—	—	—	—	—	
162	—	—	—	+++	+++	
206	+++	+++	++	+	—	
236	++	+	—	—	—	Hemorrhage
237	+++	+++	+++	++	—	
285	+++	+	—	+++	+++	
299	+++	++	+++	+++	+	Killed
299	+	—	—	—	—	Killed
299	+	+	—	+	+	Killed
321	+	—	—	—	—	

disease in untreated control animals which were inoculated in the same way and with the same dose of the same strain. In the earlier experiments, the strain of human tubercle bacilli used was of so low a virulence that some of the controls failed to develop the disease or showed only a slight development. Therefore, in later experiments, a much more virulent strain was used. In the table, "local glands" are the lymph glands near the point of inoculation, and the term "general glands" refers to lymph glands in other organs of the body. Absence

of evidence of tuberculosis in an organ is indicated by —, a small number of tubercles is indicated by +, while a very extensive process is shown by +++.

It may be seen from this table that the treatment with gold salts had no beneficial effect on the progress of tuberculosis in guinea-pigs. Life was not prolonged and the extent of the disease in the different organs was certainly not less marked in the treated than in the untreated animals. Indeed, if any distinction can be drawn, it will be in favor of the untreated control animals.

In order to determine whether the gold was well distributed throughout the animal body, my assistants Sidney M. Cadwell and Miss Gladys Leavell developed a method of analysis for the small amounts of gold found in the tissues and analyzed the tissues of a considerable number of animals, tuberculous and nontuberculous, which had been treated with the various gold salts. The details of this analytic work are presented in another paper which will soon be published.* Table 2,† however, presents figures obtained by averaging the amounts obtained in the various organs of all the different animals examined.

TABLE 2

DETAILS OF EXPERIMENTAL WORK TO DETERMINE THE DISTRIBUTION OF GOLD THROUGHOUT THE ANIMAL BODY

Average	$C_4N_4H_4Au$		$AuK(CN)_2$		$Au(CN)_3$		$AuCN$	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Days lived	130	218.6	101.7	161.5	155.5	161.5	132	161.5
Extent of disease	60%	35%	95%	95%	95%	95%	95%	95%
Mg. gold received	13.34		150.67		210.95		74.36	
Percentage								
gold excreted	40.82		17.55					
gold in organs	9.527		16.4		0.72		0.465	
gold in body	26.13		59.48					
Mg.								
found in liver	0.447		0.69		0.98		0.265	
found in spleen	0.127		0.109		0.083		0.045	
found in lungs	0.071		0.168		0.105		0.12	
found in kidneys	0.12		0.06		0.09		0.03	
found in lymph glands	0.053				0.02		0.09	
found in skin	0.45		0.27					
found in intestines	0.26		0.69					
found in blood	0.069							
found in body	4.08		4.68					
gold per gm.								
of liver	0.0212		0.078		0.027		0.007	
of spleen	0.0916		0.1645		0.0358		0.011	
of lungs	0.0105		0.0305		0.009		0.0085	
of kidneys	0.0263		0.0087		0.0165		0.007	
of lymph glands	0.0246				0.005		0.027	
of heart	0.009							
of blood	0.006							
of skin	0.027		0.0247					
of intestines	0.0075		0.013					
of body	0.012		0.0145					

*Accepted for early publication in the Journal of Pharmacology.
†Reprinted from the American Review of Tuberculosis, 1917, 1, p. 424.

It will be seen from Table 2 that the gold is not uniformly distributed throughout the body, the spleen containing more per gm. weight of the organ than any of the other organs. This may be due to the fact that the spleen is the organ in which red blood cells are for the most part destroyed, and these may easily carry with them an excessive amount of gold. In cases in which the brain was examined, no gold was found in it, and usually none in the blood except for a very short time after injection. If we examine the amount of gold found per gm. weight of the organs, we can readily see that even the smallest concentrations are far greater than the 1 : 2,000,000 generally accepted as the concentration required to inhibit in the test tube the growth of tubercle bacilli, while the amount in the spleen at least is more than sufficient to equal the concentration 1 : 20,000-30,000 stated by Feldt to be sufficient for complete inhibition in blood serum. Yet the spleen is the organ earliest and most severely involved in the tuberculous process. It is difficult to explain the fact that gold salts are so ineffective in the body of the guinea-pig, although found there in concentrations more than sufficient to inhibit growth, unless we assume that in these organs the gold is fixed in the cells and no longer has the power to act on the bacilli.

The main pathologic lesions found in both tuberculous and nontuberculous animals treated with gold salts are hyperemia of all the organs and fat infiltration of the liver and kidney. Hemorrhages were by no means uncommon, sometimes from the mesenteric vessels and sometimes from thoracic vessels. Frequent small hemorrhagic foci were found in the lungs and occasional hemorrhagic infarcts. In some cases I have noted zones of hemorrhage surrounding tubercles, but I have not found them so common as noted by Spiess and Feldt.

In reports on the efficacy of gold in the treatment of tuberculosis, as indeed is true of all other treatments of tuberculosis, we generally find the statements made that the treatment prolonged life, increased weight, caused scarring of tubercles, etc. Table 1 shows clearly that the duration of life in tuberculous guinea-pigs, whether treated or untreated is very variable, while Table 2 demonstrates that the average duration of life of the controls is greater than that of the treated animals. In any single series of animals, unless a very large dose of a very virulent culture is administered or unless intravenous inoculation is practiced, we will find certain animals living very much longer than the others, even though all received the same dose of the same culture. Thus in one series of guinea-pigs inoculated Jan. 26, 1915, one died May 26, another June 8, another June 23, another July 12, another

July 19, while one lived until Nov. 15, 1915. Lewis noted a similar lack of uniformity in the duration of life in sets of animals receiving the same inoculation. Hence we may say that prolongation of life, unless it is consistent and marked, cannot be taken as an index of the value of any therapeutic agent. As to the weights, it is stated that the controls constantly diminished in weight while the treated animals increased. My experience has been that, unless a massive and overpowering injection is given, practically all animals will increase in weight for a time, beginning to diminish in weight only when the disease has progressed to the point where general intoxication is overpowering the animal and death is imminent. The question of weight, then, depends on dose and virulence of the organism rather than on treatment.

TABLE 3

DAYS OF LIFE AND WEIGHTS OF A SET OF GUINEA-PIGS GIVEN LARGE DOSE OF VIRULENT TUBERCLE BACILLI

	Treated with Aurocantan					Controls				
	1	2	3	4	5	1	2	3	4	5
Days of life	92	51	57	37	7	105	105	27	104	59
Weight, gm.:										
1st week	530	615	525	240	355	580	730	590	295	240
2nd week	520	585	500	230		620	720	650	315	240
5th week	530	615	505	260		625	675	680	335	260
7th week	520	595	510	230		650	630	660	315	240
9th week	520	595	565	230		670	640		355	280
11th week	475	585	555	225		645	645		365	285
13th week	465	585	575			670	645		385	290
15th week	445	580				685	645		403	257
17th week		545				630	510		395	205
21st week		560				635	580		410	
23rd week		525				655	590		430	
25th week		495				650	570		385	
27th week		445				675	570		360	
29th week						610	500			

Table 3 gives the days of life and the weights throughout life of a set of guinea-pigs which received a rather large dose of a virulent culture of human tubercle bacilli. In this set the weights were more irregular and diminished more than with smaller doses of less virulent organisms. Even in this set, the weights, as well as the duration of life were better in the controls than in the treated animals. Furthermore, I have never seen any tendency to connective tissue encapsulation or scarring of tubercles such as is described by many writers.

From my experiments then, the conclusion must be drawn that the gold salts used are not efficacious in the treatment of experimental tuberculosis in guinea-pigs. No attempt has been made to use these salts in human patients and we have no wish to draw any unfavorable conclusions as to the efficacy of gold salts in the treatment of human tuberculosis. But the tendency to hemorrhage and hyperemia in the

gold treated animals and the marked shortening of life on account of the treatment certainly suggest that the treatment of human tuberculous patients with gold is not without danger. Furthermore, the chemical analyses of the organs demonstrate that there is no specific affinity of gold for tuberculous tissues and that the gold in the tissues is probably fixed in the tissue cells in such a form that it cannot inhibit the growth of the tubercle bacillus nor the development of the tubercle even when its concentration in the tissues is much more than sufficient to cause complete inhibition in the test tubes.

BIBLIOGRAPHY

Abu Moussa Diafar: Reviewed in "The Healing Art," 1887, 1, p. 145.
Bacon, Roger: Reviewed in Woolton, Chronicles of Pharmacy, 1.
Behring: Ztschr. f. Hyg., 1890, 9, p. 395.
Bettmann: München. med. Wchnschr., 1914, 5, 50, p. 798.
Blendberger: Dermat. Wchnschr., 1914, No. 6.
Bruck and Glück. München. med. Wchnschr., 1913, 60, p. 57.
Burnett: Reviewed in Woolton, Chronicles of Pharmacy, 1.
Busquet: Compt. rend. Acad. d. sc., Paris, 1915, 160, p. 404.
Cadwell: In press, Jour. Am. Chem. Soc., 1918.
Cadwell and Leavell: In press, Jour. Am. Chem. Soc., 1918.
Chrestien: Recherches et observations sur les effets des preparations d'or, 1810. Reviewed by J. B. White, loc. cit.
DeWitt: In press, Jour. of Pharmacol., 1918.
DeWitt and Sherman: Jour. Infec. Dis., 1914, 15, p. 245.
Ebn Sina: Reviewed in Sprengel's Arzneikunde, 1800.
Geber: Gyógyázat, 1913; abstr. in Internat. Centralbl. f. d. ges. Tuberkul. Forsch., 1913-1914, No. 8, p. 337.
Gibbes and Shurley: Therap. Gaz., 1892, 16, p. 235.
Hahnemann: Reviewed in Woolton, Chronicles of Pharmacy 1.
Hauck: München. med. Wchnschr., 1913, 60, p. 1824.
Heubner: Arch. f. exp. Path. u. Pharmakol., 1907, 56, p. 370. Deusch. med. Wchnschr., 1913, 39, p. 690.
Junker: Ztschr. f. ärztl. Fortbild., 1913, 10, p. 715. München. med. Wchnschr., 1913, 60, p. 1376. Beitr. z. Klin. d. Tuberk., 1914, 7 suppl., p. 421.
Keeley: Jour. Am. Med. Assn., 1907, 49, p. 1861.
Koch: Deutsch. med. Wchnschr., 1890, 16, p. 757.
Lecoque: Reviewed in Woolton, Chronicles of Pharmacy, 1.
Lewis: Harvey Lectures, 1916-1917.
Marsalongo and Vivaldi: Riforma med., Rome, 1917, 33, p. 397; abstr. in Bacteriol. Abstracts, 1917, 1, p. 388.
Merrill: Med. News, Phila., 1893, 62, p. 378.
Mayer: Deutsch. med. Wchnschr., 1913, 39, p. 1678. Beitr. z. Klin. d. Tuberk., 1914, 32, p. 211.
Oberstadt: Internat. Centralbl. f. d. ges. Tuberk. Forsch., 1917, 40, No. 1. Abst. in Am. Rev. of Tuberculosis, 1918, 1, p. 737.
Paracelsus: Reviewed in The Healing Art, 1887, 1, p. 145; Woolton, Chronicles of Pharmacy, p. 1; Sprengel's Arzneikunde, 1800.
Pasini: Gior. ital. d. mal. ven., 1914, No. 4.
Pekanovich: Internat. Centralbl. f. d. ges. Tuberk.-Forsch., 1913-1914, 8, p 337
Pereira: Materia Medica, 1839-1840.
Perutz and Seppel: Ztschr. f. Laryngol., 1913, 6, No. 5. Ztschr. f. Chemotherap., 1914, 3, p. 317.
Poor: Deutsch. med. Wchnschr., 1913, 39, p. 2303. Gyógyászat, 1913; abstr. in Internat. Centralbl. f. d. ges. Tuberk. Forsch., 1913-1914, No. 8, p. 337.
Richards: Proc. Soc. Exp. Biol. and Med., 1903, 1, p. 21.
Rosenthal: Bull. gén. de thérap., Paris, 1913, 165, p. 804. Ibid., 1914, 167, p. 75. Arch. gén. de méd., 1913.
Schumacher: Dermat., Ztschr., 1915, 22, p. 10.
Schoenfeld: Dermat. Wchnschr., 1914, 58, p. 599.
Shiga: Jour. Bacteriol., Tokio, July 10, 1916, No. 250.
Spiess and Feldt: Deutsch. med. Wchnschr., 1914, 40, p. 579. Beitr. z. klin. d. Tuberk. 1914, 30, p. 324. Berl. klin. Wchnschr., 1915, 52, p. 365.
Feldt: München. med. Wchnschr., 1914, 61, p. 1455. Deutsch. med. Wchnschr., 1913, 39, p. 549.
Spiess: München. med. Wchnschr., 1914, 61, p. 842.
Sprengel: Arzneikunde, 1800.
Takenaka: Mitth. med. Gesellsch., Tokio, 1917, 31, p. 595.
Walther: Arch. f. Dermat. u. Syph., Suppl. 117, No. 5.
White: Am. Med. Surg. Bull., N. Y., 1894, 7, p. 388. Med. Rec., N. Y., 1891, 39, p. 338.

FOCAL DEGENERATION OF THE LUMBAR CORD IN A CASE OF INFANTILE SCURVY

Alfred F. Hess

NEW YORK

The number of necropsies on cases of infantile scurvy has not been large. It is a disorder for which we possess a most efficient cure, so that the mortality is low, and the deaths are generally due to some intercurrent disease. It is remarkable to note in reviewing the necropsy protocols, how intensely the interest has been centered on the osseous system. We find the pathologist devoting page after page to the minutiae of the changes in bones, and in most instances either failing to mention the other organs, or passing them by with scant notice. As far as I am aware, there has been no microscopic examination of the nervous system in a case of infantile scurvy. A study of this kind would seem to be indicated in view of the similarity in many of the clinical symptoms between this disease and beriberi, which is classed by some among the diseases of the nervous system. Furthermore, nervous manifestations have been noted in the course of infantile scurvy, such as tachycardia, changes in the knee reflexes, alterations in the optic disks,[1] etc., so that it seemed well worth while to investigate whether the nervous system was involved in this disorder.

Bertha H., aged 18 months, was born and brought up in the Misericordia Hospital of this city. She had never been nursed, but was fed on pasteurized milk, and in addition received cereal gruels. Jan. 3, 1917, she was transferred to the Willard Parker Hospital for infectious diseases, on account of a nasal culture showing the diphtheria bacillus. The throat culture was negative, and there were no clinical signs of diphtheria. She also had marked rickets and scurvy. The latter disease was evident from the subperiosteal swelling of the left humerus, accompanied by a separation of the epiphysis, and swelling of the forearm, from the swelling and tenderness of the right thigh, the edema of both legs and feet, and from a subcutaneous hemorrhage of the eyelid.

She had received orange juice and beef juice for some days, and this treatment was continued during the stay at the hospital. However, the temperature was 103-104 F., and soon definite signs of pneumonia developed, of which disease she died Jan. 14. The child was poorly developed and had a marked

Received for publication May 21, 1918.

[1] Hess, A F.: Jour. Am. Med. Assn., 1917, 68, p. 235.

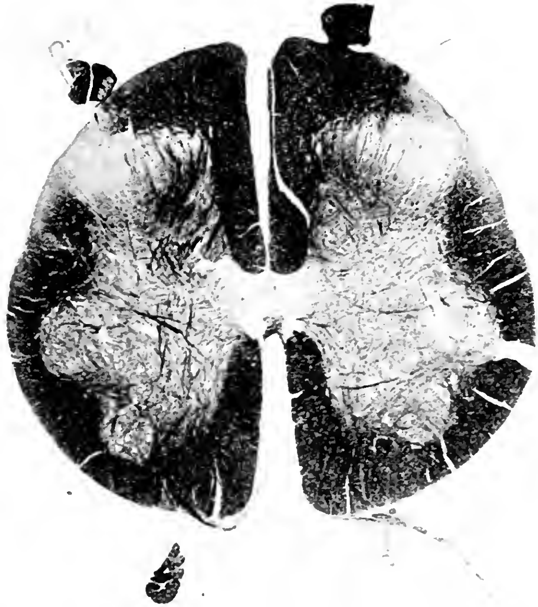


Fig. 1.—Low power. Showing pale area and paucity of cells and fibers in lateral group of left anterior horn. (Frozen section, Spielmeyer's iron alum hematoxylin method.)

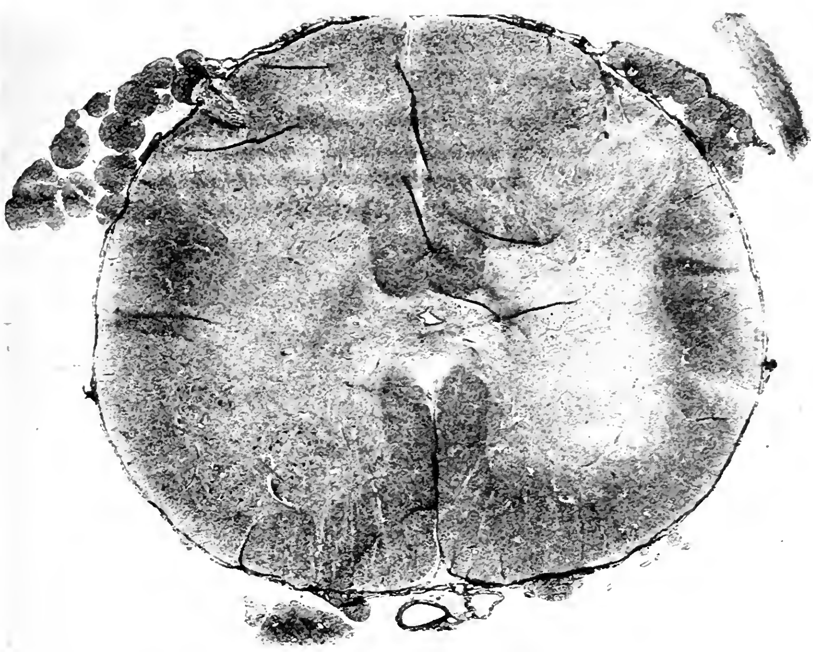


Fig. 2.—Lower power. Showing same as previous figure. (Marchi stain, vital scharlach counter stain.)

rachitic rosary. The plasma coagulation time was normal (6 minutes), bleeding time normal, "capillary resistance test" negative, puncture test positive.

At necropsy, separation of the epiphysis of the left humerus was found. There had evidently also been an infraction of the head of the right femur, so that it was depressed, and assumed a position almost at right angles with the shaft of the bone—a condition closely resembling that of coxa vara.* There were petechial hemorrhages in the pericardium with some slight increase of its fluid contents. The heart was slightly enlarged, the lungs showed a considerable pneumonic involvement of the right upper and left lower lobes; the abdominal viscera showed no changes excepting a few petechiae in both kidneys.

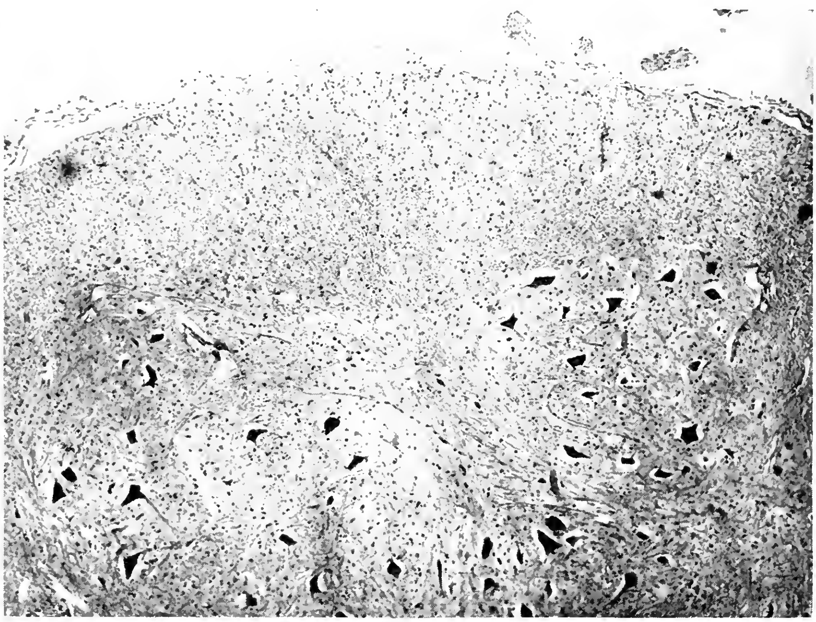


Fig. 3.—High power. Showing normal cells in lateral group of right anterior horn.

As mentioned, special attention was given to the spinal cord and peripheral nerves. The tissues were preserved in formalin, and were prepared by Dr. Charles B. Dunlap of the Psychiatric Institute of the New York State Hospital, whom I take this opportunity to thank for his painstaking work in this connection. The following is a summary of his report:

Spinal Cord: Sections were stained with toluidin blue, hematoxylin and eosin, Weigert's neuroglia, Marchi, scharlach R. and myelin sheath stains.

The nerve cells and neuroglia in the cervical part were normal in appearance except for a slight excess of neuroglia cells, and some bunching of these in minute foci. There were no changes in the middorsal cord. In the lumbar

* This lesion of coxa vara in association with infantile scurvy is of interest in view of the possibility that it may, in some instances, be the cause of this malformation.

cord there was a striking local loss of nerve cells in the lateral groups of the left anterior horn. A pale area was seen at this site containing numerous irregular nuclei. The preserved nerve cells were in fair condition. The pia contained more cells than usual, but not a pronounced exudate. The neuroglia was about equal on both sides. The Marchi stain was negative, but scharlach R. showed abundant fat droplets in the pale area and along the course of the anterior root fibers. There were fewer nerve fibers in the pale area than on the right, but less difference than one would expect. At slightly different

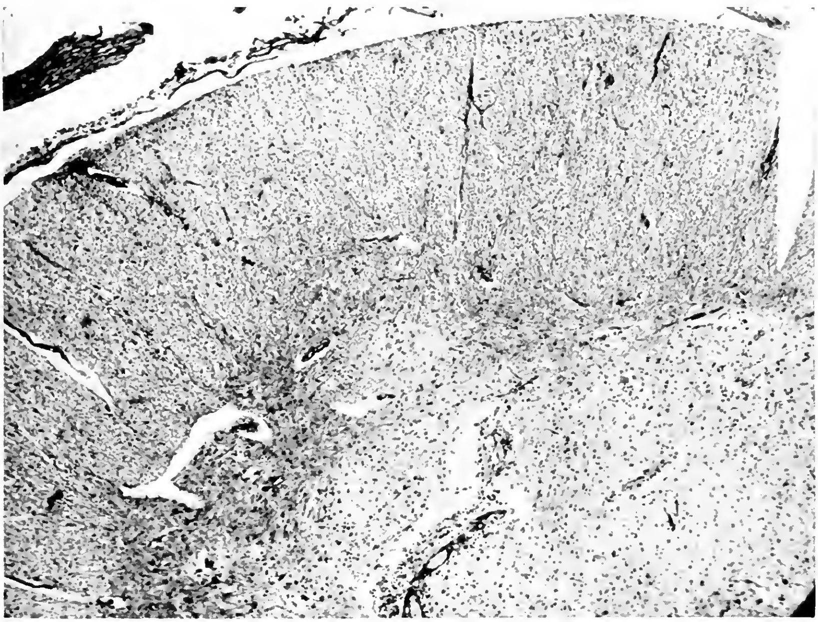


Fig. 4.—High power. Showing focal degeneration and absence of cells in lateral group of left anterior horn.

levels on the left, the nerve cells increased, indicating the local nature of the lesion. The cell counts on the two sides, as observed with various stains, were as follows:

	General Averages	
	Lesion Side	Sound Side
Alcohol	11	50
Neuroglia	11	34
Weigert sheath...............	26	36
Marchi	11	43

Brachial plexus was normal except for pale staining of the myelin sheaths.

Transverse and longitudinal sections of the sciatic nerve failed to show any changes except pallor. There were numerous bundles—more than 50.

The pneumogastric nerve, upper portion, in transverse and longitudinal sections appeared normal except for pallor of the nerve sheaths, and many small rings in which no axis cylinders were seen. In the lower portion was possibly a slight exudate in the epineural sheath and connective tissue.

The examination may be summarized by the statement that the only definite change found was a focal degeneration of the lumbar cord, which extended for a distance of perhaps a quarter inch. We have no means of definitely interpreting these changes. That they were not the result of poliomyelitis would seem evident, not only on account of the noninflammatory nature of the lesion with its absence of round cell infiltration, and lack of characteristic changes in the remaining anterior horn cells, but also in view of the fact that the child had never shown any symptoms of this disease, and had been brought up in an institution where no instance of infantile paralysis had developed. It would seem to belong rather to the degenerative type of lesion. Whether it is truly scorbutic in nature or merely happened to be associated with this disease, only an examination of other cases can decide. In some respects it resembles the microscopic lesions of lead poisoning, described by Oppenheim and Monakow, and similar lesions reported in experimental lead poisoning by Stieglitz.[1] In this connection it is of interest to remember that Eijkmann,[2] in his classic article first describing the polyneuritis of fowl, mentions changes in the cord, "especially degenerative and atrophic changes in the ganglion cells of the anterior horns," and that Shiga and Kusama[3] substantiated this observation by finding a similar atrophy of the ganglion cells of the anterior horns. My case is reported rather to stimulate further investigation than to pronounce definite judgment on the proper interpretation of the cord lesion.

[1] Lewandowsky: Handb. d. Neurologie, 1911, 2, p. 55.
[2] Virchow's Arch., 1897, 148, p. 523.
[3] Beihefte z. Arch. f. Schiffs- u. Tropen-Hyg., 1911, 15, Part 3.

RELATIONSHIP OF THE LEUKOCYTE COUNT AND BONE MARROW CHANGES IN ACUTE LOBAR PNEUMONIA

S. S. Samuels and Robert A. Lambert

From the Pathological Laboratory of the Presbyterian Hospital, New York

It is well recognized that the leukocyte count in lobar pneumonia may vary within wide limits. The majority of the cases which end fatally show either a very high or a relatively low count, while those with favorable outcome most often have counts between these extremes.[1] These points are brought out rather strikingly in the accompanying chart, showing a comparison of the leukocyte counts in 26 fatal and an equal number of recovered cases of acute lobar penumonia.

The cases with persistently low leukocyte counts may be considered first. At least two explanations for these relatively low counts have been suggested: (1) The bone marrow fails to react, either as the result of some previous injury (chronic alcoholism, for example), or on account of a paralysis of the blood-forming elements from over-stimulation induced by the pneumococcus infection itself. (2) A rapid spread of the pneumococcus process results in the withdrawal of leukocytes from the blood faster than they are thrown into the circulation from the bone marrow, so that the number of circulating leukocytes may be normal or only slightly increased, in spite of the fact that the output of the bone marrow factory is far above normal. Still another possibility must be conceived, namely, that leukocytes may be formed in some other organ than the bone marrow, for example, the spleen. F. A. Evans,[2] working in this laboratory, pointed out several years ago that the gray, acute splenic tumor of lobar pneumonia contained large numbers of cells of the myeloid series, as indicated by the oxidase reaction, and suggested that the spleen might be the source of certain of the cells of the pneumonic exudate.

Received for publication June 3, 1918.

[1] Osler: Principles and Practice of Medicine, Ed. 8, p. 87.

[2] Bull. Johns Hopkins Hosp., 1916, 27, p. 356.

The first explanation for the leukocyte count, namely, that the bone marrow fails to react, has been discussed by Muir, Longcope, Dickson, and others. In most of these papers the subject of acute lobar pneumonia has been considered secondarily, in connection with types of acute, nonsuppurative infections.

Muir,[3] in discussing the reaction of the bone marrow and other leukocyte-forming tissues in infection, found that the marrow pictures in his cases of acute pneumonia varied considerably. These variations he attributed to such factors as the age of the patient, the hyperplasia being less marked in late years; to the duration of the disease; to the severity of the toxemia, and to possible unknown causes such as the resisting power of the individual. He ascribed the phenomenon of depletion of adult polymorphonuclear leukocytes from the marrow to an emigration of these cells from the marrow just before death, probably in the agonal stage.

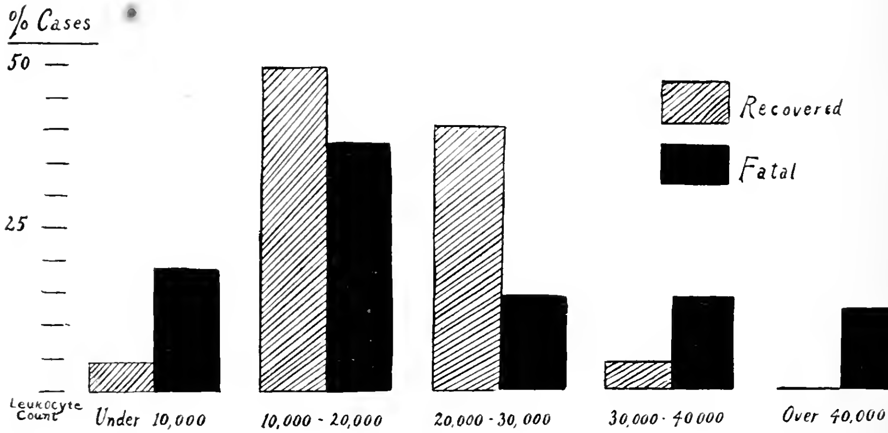


Chart 1.—Comparison of the leukocyte counts in 26 fatal and an equal number of recovered cases of acute lobar pneumonia.

The latter point was investigated experimentally by Longcope.[4] He worked with inoculated rabbits and studied their bone marrow during the course of the disease and postmortem. He explained the disappearance of the polymorphonuclear leukocytes from the marrow in any acute infection as due to a direct toxic action of the infection on the marrow, rather than to a simple emigration of the cells.

In another paper, Longcope[5] cites 2 cases of acute pneumonia in which the leukocyte count during life was below normal and the necropsies revealed an

[3] Jour. Path. and Bakteriol., 1901, 7, p. 161; Tr. Path. Soc., London, 1902, 53, p. 379.
[4] Bull. Ayer Clin. Lab. of Penn. Hosp., 1907, 4, p. 6.
[5] Bull. Ayer Clin. Lab. of Penn. Hosp., 1905, 2, p. 1.

only a few cellular patches. His explanation of these observations is the possibility that the functional activity of the marrow cells in each of these patients was exhausted.

Schurr and Löwy[6] report 2 cases of acute pneumonia that gave constant hyperleukocytic blood counts during life and yet showed aplastic, fatty marrow necropsy. These cases were used as part evidence for disproving the importance of the bone marrow as a leukocyte-former in acute infections.

Dickson,[7] in citing cases of pneumonia with leukopenic and aplastic bone marrow, ascribes the absence of leukoblastic reaction to the fact that before or during the onset of the pneumococcus infection the individual might have been weakened by alcoholic excess, syphilis, or other similar conditions. As another cause of imperfect reaction of the marrow, he describes "gelatinous degeneration" of the blood-forming tissue. This is a change in the bone marrow brought about by a progressive diminution of its blood-forming constituents along with an absorption of the fats of the marrow. This condition may be either acute or chronic.

The object of the present study was to determine, if possible, the explanation of the striking variation in the number of circulating leukocytes among cases of lobar pneumonia. We were interested particularly in finding out whether there existed a close parallelism between the leukocyte count and the degree of hyperplasia of the marrow, as has been very generally assumed. Our observations have been made on 17 cases of acute lobar pneumonia which came to necropsy. Only adults were included in the series, since in the earlier periods of life the bone marrow in the long bones is normally so active that degrees of hyperplasia cannot be easily recognized.

In every case bone marrow was obtained from the upper third of the femur. The method of procuring the specimen has been described elsewhere.[8] The tissue was fixed in Zenker's fluid, embedded in paraffin, and stained with hematoxylin and eosin. The presence or absence of hyperplasia was determined by a careful study of the microscopic sections. Aplastic marrows were classified as negative. The hyperplastic marrows were arbitrarily divided into 5 groups, designated in the table by the following signs: ±, +, ++, +++, and ++++. The criterion for ++++ hyperplasia was an extremely cellular marrow, replacing practically all of the adipose tissue with evidence of active cell multiplication.

A detailed study of the types of cells present in the marrow was not made, although in the routine examination of the sections it was noted, that in the majority of the cases showing hyperplasia, the predominating cell appeared to be an undifferentiated marrow cell with large round, vesicular nucleus and almost entirely fatty marrow. In 2 other similar cases there was red mottling of the marrow. In 1 case with a high leukocyte count, the marrow presented

[6] Ztschr. f. Clin. Med., 1900, 40, p. 412.
[7] The Bone Marrow, 1908.
[8] Lambert, R. A.: Proc. New York Path. Soc., 1918.

nongranular or finely granular cytoplasm. Polymorphonuclear leukocytes were present in the marrow of practically every case, and in several cases they were quite numerous. In this particular, our observations disagree with those of Muir and Longcope, to which reference was made in the previous paragraph.

The accompanying table shows the sex and age of the patients, the duration of the disease, the total and differential leukocyte count during life, and the state of the marrow at necropsy. An analysis of

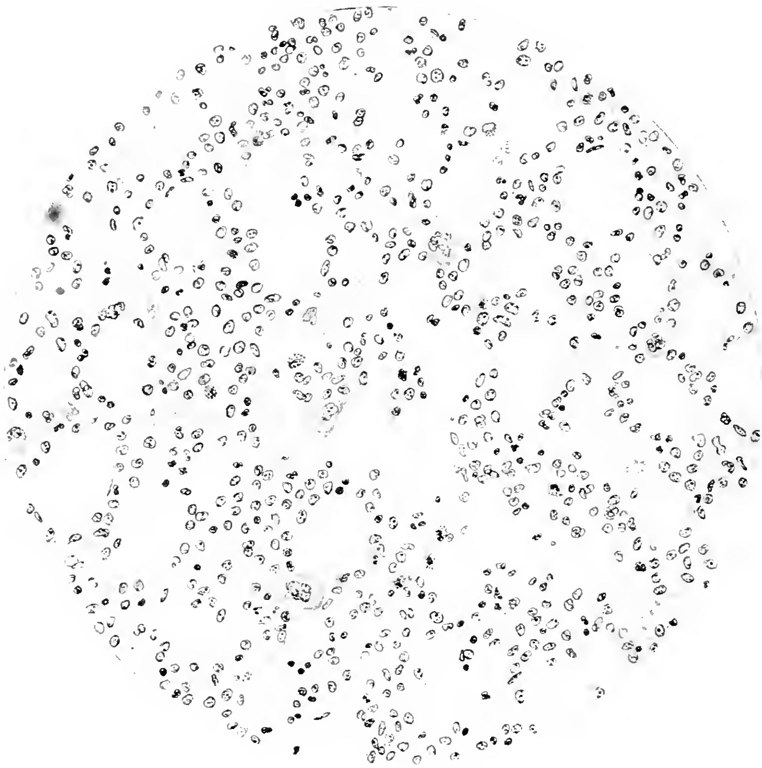


Fig. 1.—Markedly hyperplastic bone marrow from Case 17, in which the leukocyte count during life varied between 18,000 and 9,000, with a terminal count of 10,000 and 90% polymorphonuclears.

the data in individual cases gives varying results. In a few of the cases (Nos. 1, 4, 10 and 14) a close parallelism between the leukocyte count and the bone marrow changes is found. For example, in Case 4, in which the leukocyte count was persistently low, an aplastic marrow was found; and in Case 14, there was an association of high

leukocyte count and a markedly hyperplastic marrow. On the other hand, in a majority of the cases, no such agreement is seen. Indeed, several cases present a striking lack of harmony between the bone marrow and blood changes. For example, Case 17 (Fig. 1), in which there was a relatively low leukocyte count, a ++++ hyperplasia of the marrow was present. This, too, in a man of 42 years, while in Case 16, showing a moderately high leukocyte count, the bone marrow was found to be aplastic.

Fig. 2.—Aplastic bone marrow from Case 6, in which the leukocyte count during life varied between 17,000 and 21,000 with 90% polymorphonuclears.

An examination of the table shows that of the 17 cases, 11 exhibited varying degrees of hyperplasia of the marrow, and in 6 the marrow was aplastic. Of the aplastic cases, only one (Case 4) showed a persistently low leukocyte count. In another (Case 5), the count ranged from 28,000 to 41,000 during the earlier periods of the disease with a final count of only 4,000. In the other four cases, the counts averaged 20,000 with 90% polymorphonuclear leukocytes.

LEUKOCYTE COUNT AND BONE MARROW CHANGES IN LOBAR PNEUMONIA

Case	Sex	Age	Days Duration	Range		Final		Bone Marrow
				White Blood Cells	Polymorphonuclears	White Blood Cells	Polymorphonuclears	
1	M	39	30	17,000- 6,000	95-65	14,000	94	+
2	M	55	17	16,000-25,000	90-98	25,000	98	+++
3	M	71	16	48,000-29,000	94-92	29,000	92	+
4	M	27	5	9,000- 7,000	88-74	7,000	74	—
5	M	43	21	28,000-41,000	88-92	4,000	83	—
6	M	29	9	17,000-21,000	89-90	21,000	90	—
7	M	48	6	4,000-30,000	84-92	30,000	87	+
8	F	58	12	42,000-42,000	88-88	42,000	88	+
9	F	50	2	28,000-43.000	87-93	43,000	93	++
10	M	29	5	17,000- 4,000	92-55	4,000	55	±
11	M	48	7	12,000- 4,000	94-60	4,000	60	++
12	M	22	4	9,000-15,000	95-90	15,000	90	++
13	M	55	28	20,000-13,000	98-81	15,000	88	—
14	F	32	2	47,000-47,000	94-94	47,000	94	+++
15	M	53	1	29,000-29,000	99-99	29,000	99	+
16	M	29	4	19,000-27,000	92-93	27,000	93	—
17	M	42	9	18,000- 9,000	96-88	10,000	90	++++
Average	..	43	10	22,000-23,000	92-85	21,000	86	

SUMMARY

A close parallelism in the leukocyte count and the degree of hyperplasia of the marrow was found in less than half of 17 fatal cases of lobar pneumonia, which were studied. A few showed relatively inactive or aplastic marrows, with a leukocyte count well above normal. On the other hand, there were several cases in which the leukocyte count was persistently low during life, but in which a markedly hyperplastic marrow was found at necropsy.

To explain an aplastic femur marrow (a condition almost certainly common to all the long bones in these cases) associated with a leukocytosis during life, we may assume either a hyperplasia of the marrow of the flat bones only—a very improbable condition—or a formation of leukocytes outside the marrow, most probably in the spleen. The presence of large numbers of cells of the myeloid series in the splenic pulp would favor the latter explanation.

The cases of marked hyperplasia of the marrow with low leukocyte count are not easily interpreted. We could not find evidence in these cases of such rapid spread of the lesion as would account for a low leukocyte count through the draining of these cells out of the circulation.

A CASE OF SPIRILLOSIS

C. C. HARTMAN AND G. R. LACY

From the William H. Singer Memorial Research Laboratory, Pittsburgh

Much interest has recently been aroused in diseases from which curved organisms are isolated and we feel that the following case presents certain features which merit its report even though existing circumstances prevented the completion of the bacteriologic studies.

CLINICAL HISTORY

R. G., aged 22, of Italian extraction, crane operator, was admitted to the Allegheny General Hospital, Pittsburgh, April 16, 1917, service of Dr. James P. McKelvy, complaining of "stomach trouble." At this time the patient was able to walk. His family history was negligible. It appeared from his previous history, that he had been suffering from an intermittent unproductive cough accompanied by night sweats and gradual loss of weight and strength for several months, and that he had had several severe colds during the winter (1916-1917). Sore throat was not a prominent symptom of the colds. He had had gonorrhea in Dec., 1915, with good recovery. He gave no history of the usual exanthemata during childhood, but had had diarrhea at the age of 6 months which, according to his statement, had persisted for about 6 months. He denied syphilis and did not use alcohol.

The present illness began 7 months before admission with "cramps" in the region of the stomach which were aggravated by eating and lasted but a short time. As the disease progressed, the cramps became more severe after meals, varying in duration from a few minutes to several hours. The pains were relieved, as a rule, by a movement of the bowels. The stools were loose and watery. The diarrhea would entirely disappear on abstinence from food and drink. Meat particularly aggravated the symptoms. There was no bleeding, vomiting, headache or jaundice. There were no acute cardio-respiratory symptoms and the patient said there was no fever until a week before admission.

The patient did not look acutely ill. The skin and mucous membranes were pale and there was a general sallow appearance. The nutrition was below par. The eye reflexes were normal and there was no icterus of the sclerae. The mouth, ears and nose were normal. Several enlarged superficial glands were felt in the posterior cervical region, especially on the left side. The axillary lymph nodes and right epitrochlear nodes were definitely palpable. The parotid, submaxillary and thyroid glands were normal.

No definite consolidation could be found in the lungs although large and small moist râles were present in both lower axillae and in both bases.

The point of maximum intensity of the heart beat was in the 5th left intercostal space 9½ cm. from the midline. No thrills or shocks were palpable. There was a systolic murmur at the apex, slightly transmitted to the axilla. There was also a systolic murmur at the base maximal along the sternal

Received for publication June 3, 1918.

border at the pulmonic area, and well transmitted toward the neck. The heart action was regular. The pulse was regular in force and rhythm, of good volume and of fair tension. The blood pressure was 105 mm., the diastolic 40 mm., and the pulse pressure 65 mm., or 162%. The vessel walls were not appreciably thickened.

The abdomen was distended and tympanitic, the muscles were held somewhat rigidly so that the position of the organs was unsatisfactorily made out. The spleen was just palpable, the liver edge not felt. There were no masses or localized areas of tenderness. Examination of the extremities showed tenderness on pressure over the middle of the right tibia. The knee kicks were decreased. There was no edema, redness, swelling, sensory disturbances or abnormal reflexes.

The tentative diagnosis was typhoid fever. Blood cultures and agglutination tests on April 7, 14, and 21, yielded negative results. The urine was negative Culture from the urine on April 22 was negative. Blood examination on March 22 (Dr. Willetts) showed red blood cells 3,700,000; white blood cells, 9,200; hemoglobin, 63% (Sahli); differential leukocyte count — polymorphonuclear leukocytes, 63%; lymphocytes, 19%; large mononuclears, 12%. The red blood cells showed marked anisocytosis, some poikilocytosis, some anisochromia, very slight polychromatophilia, no stippling, no nucleated forms and few blood platelets. The Wassermann reaction was negative.

After some days, an area of high pitched tympany developed in the left axilla, extending somewhat anteriorly and there were signs of a diffuse bronchitis. There was an area about the apex of the heart and over the whole right base of increased breath sounds, whispered and spoken voice sounds, prolonged expiration and bronchovesicular breathing. On April 21, there was a striking enlargement of the submaxillary, posterior cervical, axillary, epitrochlear, and inguinal glands which lasted but a few days. There was no tenderness of the involved glands. The spleen became definitely palpable. There were a number of small, pea-sized nodes palpable in the anterior axillary line and along the margin of the pectoralis muscle. The temperature gradually came down to normal on April 25, and remained so until May 3. Intradermal tuberculin tests with 1/100 mg., 1/10 mg. and 1 were negative. On April 27, an acute otitis media developed on the left side necessitating incision. On April 29, an injection of 1/33 grain of atropin sulphate showed a change in pulse from about 100 to 120 per minute. On April 30, a blood count showed red blood cells, 2,000,000; white blood cells, 3,200; and hemoglobin, 30% (Sahli). On May 3, a marked swelling and tenderness on the left cheek developed which gradually disappeared under cold applications. On May 6, considerable tenderness appeared along the left femoral artery and in the adjacent muscles; no edema of the foot developed and arterial pulsation persisted. This condition cleared up after several days. A period of diarrhea then occurred, with distention of the abdomen. The fever continued, the loss of weight was marked the patient became dull and drowsy and at times obstinate and combative. The abdomen then became scaphoid and generally tender, but the reflexes remained present and active. There were abnormal reflexes in the extremities. The neck was held rather rigidly but was not painful. On May 14, a blood count showed red blood cells, 848,000; white blood cells, 1,500; hemoglobin, 10% (Sahli). The red blood cells showed considerable anisocytosis, poikilocytosis and slight anisochromia, but were generally of a good color. No abnormality was noted in the white blood cells except perhaps an increase in the number of mononuclear cells. No pigmented cells were seen. On May 10, a blood culture was taken, which after 5 or 6 days'

incubation showed the presence of a very actively motile curved organism. On May 14, the patient died.

Cultures taken during the necropsy yielded organisms identical with those isolated before death.

BACTERIOLOGIC EXAMINATION

The organisms isolated before death appeared in dextrose broth, serum broth and in deep agar cultures. Culture of the heart blood at necropsy

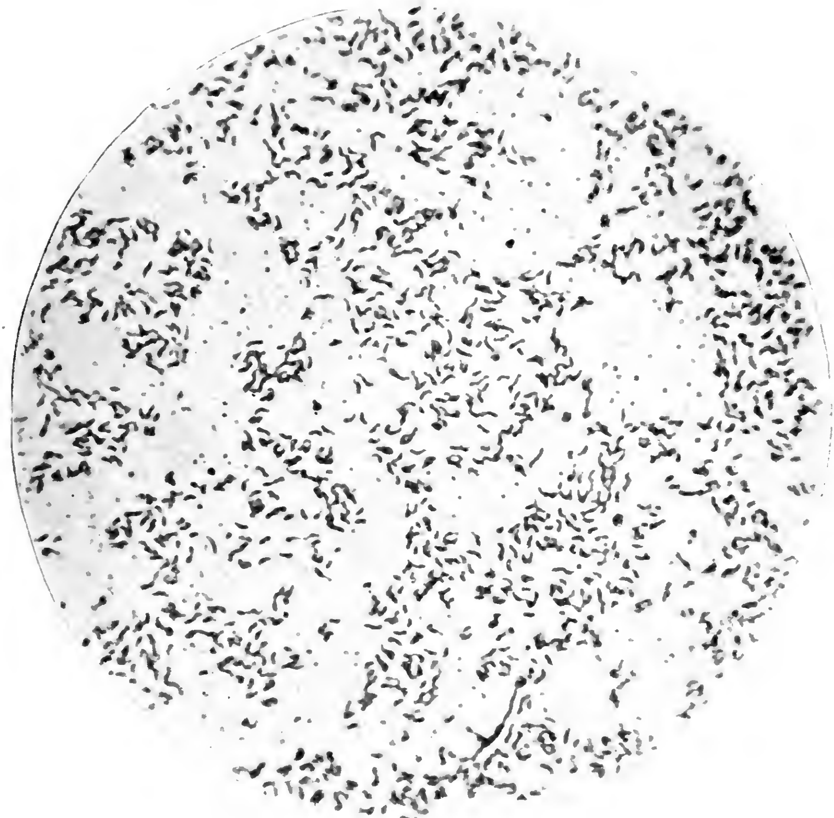


Fig. 1.—Comma-shaped organisms from freshly isolated cultures.

yielded similar organisms in dextrose broth, lactose broth, Noguchi tissue broth and on blood agar. Cultures from the spleen, lymph nodes, urine and pleural fluid were negative.

Transfers were made from these fluid cultures to plain agar, blood agar and Loeffler's blood serum. No growth occurred on plain agar, but on the 3rd or 4th day, very fine moist colonies were found on the Loeffler serum and blood agar. The organism grew rapidly well under anaerobic and aerobic conditions. After being kept on solid mediums for a few weeks the cultures became

luxuriant and colonies were visible in 48 hours after transfer. It was necessary to plant them every 10 days or 2 weeks to keep the cultures living. Transfers made to carbohydrate broths and milk at this time showed a very slight amount of growth. After 6 months (May-October) transfers made to the same mediums produced in 48 hours a marked turbidity but no acid or gas. After 2 weeks on the litmus milk there occurred peptonization and precipitation without any change in the reaction and without coagulation. No indol was formed.

Fig. 2.—Well developed spirilla found after several transplants on artificial mediums.

Microscopically, the organisms were interesting because of the changes in morphology which occurred from time to time. In the freshly isolated cultures they appeared as short, commalike bodies, having from 1-3 curves (Fig. 1). After a few transfers, a predominance of long, slightly spiral, threadlike bodies were found (Fig. 4), while subsequent transfers showed types, varying from the short vibrios to the long threadlike forms, with many intermediate stages (Fig. 3). In some cultures definite spirilla were found (Fig. 2).

Inoculations of rabbits, guinea-pigs, white rats and white mice failed to throw any light on the character of the organism. It is probable that repeated inoculations, or the use of a greater variety of animals, would have been of value. Forty-eight hour growths on blood-agar slants were washed off with salt solution and the growth from a single slant used as the dose. Full doses were given to each of 3 rabbits intravenously, and in 4 guinea-pigs and 1 white rat intraperitoneally. Half doses were injected intraperitoneally into

Fig. 3.—Cultures showing various types.

2 white mice. All of the animals were observed for a period of more than 6 weeks and none of them showed any untoward effects.

POSTMORTEM EXAMINATION (DR. HAYTHORN)

The body was greatly emaciated, severely anemic, with general glandular enlargement. About 300 c c of clear fluid present in the left pleural cavity. The right lung adherent. Both pleural surfaces covered with petechial hemorrhages. Both lungs pale, edematous, and contained healed tubercles. The peribronchial lymph nodes anthracotic and contained healed tubercles. The

pericardial sac contained over 100 c c of clear fluid. The epicardium smooth and glistening. The subepicardial fat showed serous atrophy. The right side of the heart dilated; myocardium pale and edematous. The endocardial surfaces and valves normal. The aorta was surrounded with a mass of grayish-brown lymph nodes, firm and fibrous. The aortic wall thickened, inelastic and showed irregular linear intimal sclerosis, most marked in the arch. The abdominal cavity contained 2000 c c of clear straw colored fluid. The fat was almost entirely absent. The lower border of the liver was 10 cm.

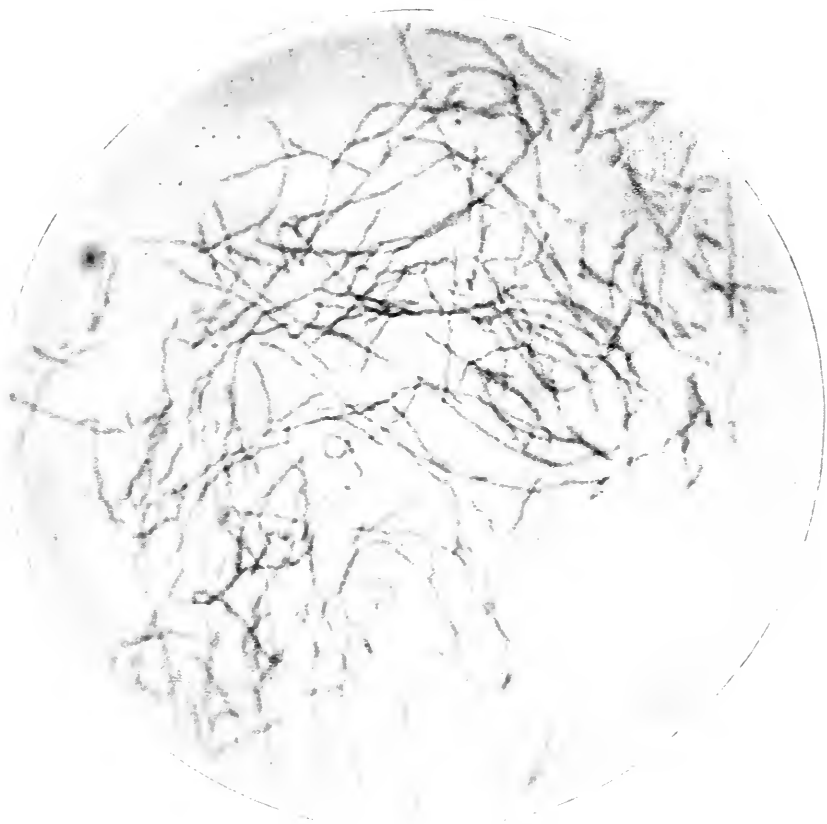


Fig. 4.—Threadlike forms developed after several days on artificial mediums.

below the costal margin. Adhesions between the colon and gallbladder. The mesenteric and retroperitoneal lymph nodes enormously enlarged. The spleen weighed 1,160 gm. and contained multiple infarcts. The pulp soft and purplish-red in color. The splenic artery and vein free from thrombi and sclerosis. The mucous membrane of the stomach was pale and many minute superficial more or less puckered ulcers were present. The small intestine showed nothing of interest. The colon was covered with a finely granular exudate and the walls were edematous. The liver weighed 1,920 gm. The

capsule showed many white linear thickenings; the cut surface mottled, pale tan and white, with some hemorrhagic spots. The gallbladder and ducts normal. The pancreas weighed 110 gm. and was edematous. The adrenals normal. The kidneys weighed 220 and 200 gm., respectively; capsules adherent; the cortical surfaces showed a few petechial hemorrhages; the cortex pale and swollen; the glomeruli seen with difficulty. The pelvic mucosa contained petechial hemorrhages. The other genito-urinary organs showed no particular changes. The marrow in the vertebrae was semisolid and of a magenta red color; that of the femur bright red, hemorrhagic and soft; there was no marginal area of spongy bone and no evidence of bony absorption.

Microscopically the heart muscle cells were markedly infiltrated with fat granules and a general degenerative myocarditis was present. The aorta presented a marked intimal and subintimal sclerosis with destruction of the elastic layers and connective tissue replacement. There was edema and anthracosis of the lungs with fresh hemorrhagic foci on the pleura. Sections from the ulcerated areas of the stomach were in general suggestive of those of the intestine in early typhoid fever: They were characterized by destruction of the glands in the lower layers of the mucosa accompanied by both hyperplasia and infiltration of endothelial cells. The latter were phagocytic for red cells, cellular débris and chromatin substances. The submucosa was also invaded but the muscular layers apparently free from inflammatory exudate. The pancreatic trabeculae were infiltrated with lymphocytes. The liver was edematous, the cells greatly compressed and elongated. The sinusoids were dilated and free from red cells. The periportal connective tissue appeared much compressed and thickly infiltrated with lymphocytes, plasma and endothelial cells. The lobules were distended and the peripheral portions seemed to be fairly well preserved. Some of the cells gave a faint reaction for hemosiderin. The central and midzonal areas contained foci of necrosis in which the liver cells had either entirely disappeared or were decreased in number. Such foci were extensively infiltrated with phagocytic endothelial cells, lymphocytes, plasma cells, and a moderate number of neutrophils. The endothelial cells contained entire cells, cellular débris and comma-shaped bits of material believed to be spirilla. Cocci were present in small numbers. The process, while generally focal, was also more or less diffuse. Many of the endothelial cells gave a very decided iron reaction, and all of the remaining liver cells gave a strong red reaction for granular fat with sudan III, A few focal necroses were present in the adrenals. The kidneys were edematous; the tubular epithelium atrophied and reacted positively for fat; hyalin and blood casts were present. The testes were edematous. The spleen was congested and infarcted. Several thrombosed vessels were present and the general picture was that commonly seen in acute septic conditions. Nestlike phagocytes filled with red blood cells and débris were abundant and questionable comma-shaped organisms numerous. The marrow was largely made up of a waxy homogeneous nonnucleated material; where less injured marrow persisted the tissue was infiltrated with phagocytes. Many bacteria, either short chains of cocci or spirilla, were found both within cells and free in the substance. No normal areas were found. The lymph nodes presented granulomatous changes, the germ centers being destroyed and replaced by a loose vascular stroma, which was more or less hyaline in places; the tissue was infiltrated with large single and multinucleated cells (having from 2-6 nuclei) which showed no necrosis and were intensely phagocytic for cells, débris and spirilla. The process was suggestive of, but not identical with, the

Dorothy Reed type of Hodgkins disease. The findings in the nodes may be summarized briefly as that of a diffuse sclerosing process with tumorlike giant cells caught in the new formed tissue meshes.

SUMMARY

The occurrence, in a young Italian, of a recurrent fever characterized by acute exacerbations, accompanied by severe secondary anemia, general lymph node involvement, splenomegaly and varying lung symptoms, and the isolation from the blood both during life and at necropsy of a motile curved organism are the salient features of the case.

The resemblance to typhoid fever during the earlier course of the disease was striking save that all of the laboratory tests were consistently negative. The next tentative diagnosis was that of a generalized tuberculosis, but this was not corroborated by tuberculin tests and further observations. The most unusual feature was the sudden enlargement of the lymph nodes with subsidence within a few days to a more nearly normal size. A study of the sections suggests that enlargement .was due to a severe inflammation of the intranodal sinuses which healed by granulomatous changes leading to extensive connective tissue development. The progressive secondary anemia was extreme, and there were no evidences of blood regeneration found either in the blood examinations or in the sections of the marrow. The marrow was red but the sections showed the color to be due to necrosis and hemorrhage and not to hyperplasia. The hemosiderin in the liver and spleen as well as the extreme phagocytosis of red cells in the latter showed that the anemia was due not only to the failure in production of red cells, but also to actual blood destruction in the tissues. The splenomegaly was probably secondary to the anemia. A few small foci of nodular connective tissue, slight edema and the pleural hemorrhages were all that were present at necropsy to explain the lung symptoms. Anatomic changes associated with the diarrhea and general toxic condition were seen in the stomach ulcers, the membrane in the colon and in the necroses of the liver. The main point of interest is, of course, the isolation both before and after death of an actively motile, spirally curved organism from the blood. This spirillum grew equally well aerobically and anaerobically. Just where it should be classified among spirally curved organisms we are not prepared to say.

LIBERATION OF ANTIBODIES ON INJECTION OF FOREIGN PROTEINS *

SIEGFRIED F. HERRMANN

From the Department of Bacteriology, University of Minnesota, Minneapolis

Many reports have been published concerning the treatment of acute and chronic infections by the intravenous injection of foreign proteins. This treatment has been applied to typhoid fever, arthritis, and certain skin diseases. It apparently developed as an outgrowth of the injection of vaccines. Vaccine therapy, once the great hope of modern therapeutics, failed to produce the promised results, and, with the exception of prophylactic vaccination against typhoid, variola, rabies, and perhaps in acne and allied pyogenic skin diseases, was being abandoned more and more. Now there is an apparant revival of allied therapeutic methods in the form of foreign protein injections.

The beginning of foreign protein therapy dates back to the work of Fraenkel[1] in 1893, who treated 57 cases of typhoid fever with subcutaneous injections of typhoid vaccine. He reported that most of the cases were favorably modified, and that a few terminated by rapid lysis. This use of typhoid vaccine during the active, acute stage of the disease was a new departure, which demanded further investigation.

Rumpf,[2] using a vaccine of Bacillus pyocyaneus, repeated the work in another group of cases, and observed equally favorable results.

Ichikawa,[3] using intravenous injections of sensitized vaccine, and Kraus and Mazza,[4] using polyvalent vaccine, were able to produce favorable modifications in 60% of their cases. They also observed equally good results in paratyphoid and found that colon vaccine could be substituted for the typhoid vaccine.

More recent work on typhoid has been reported by Lüdke[5] with albumoses; Saxl,[6] using a solution of caffein and camphor; Kibler and McBride,[7] using typhoid vaccine, etc.

Foreign protein has been used also in the treatment of arthritis. Notable is the report of Miller and Lusk.[8] Miller later summarizes his results and

Received for publication June 3, 1918.

* Supported by grant from the National Dental Research Association.

1 Deutsch. med. Wchnschr., 1893, 19, p. 985.
2 Ibid., 1893, 19, p. 987.
3 Ztschr. f. Immunitätsforsch. u. exper. Therap., 1914, O., 23 p. 32.
4 Deutsch. med. Wchnschr., 1914, 40, p. 1556.
5 München. med. Wchnschr., 1915, 62, p. 321.
6 Wiener med. Wchnschr., 1916, 66, p. 115.
7 Jour. Infect. Dis., 1917, 21, p. 13.
8 Jour. Am. Med. Assn., 1916, 66, p. 1756 and 1916, 67, p. 2010.

gives an extensive bibliography of work done by other clinicians.[9] The results as reported appear promising, especially in acute arthritis.

Other favorable results have been reported in skin diseases, puerperal sepsis, conjunctivitis, iritis, trachoma, typhus, tuberculosis, etc. Among the variety of proteins employed are: colon vaccine, chicken serum, whole blood, proteose, albumose, horse serum, and autolyzed vaccine.

Closely related to the response to foreign protein is the old and familiar observation that a specific disease may sometimes improve with the advent of some intercurrent infection. For example, the symptoms of acute gonorrhea have been observed to subside with the incidence of typhoid fever. Attention has also been called to the increased resistance of the body to various common infections, following any vaccine injection.

It is evident that foreign protein therapy is empirical and non-specific. Almost any foreign protein seems to be effective, and may be employed more or less successfully, under suitable conditions, in a variety of diseases. The question arises as to what factor is responsible for the apparent benefits observed. If this manner of treatment is to be placed on a scientific basis this factor must be determined. Clinical investigations have thrown no light on this question. The type of reaction is apparently the same in all cases.

As described by Kibler and McBride[7] the reaction of a typhoid patient to the intravenous injection of typhoid vaccine is as follows:

1. *Temperature.*—Within a few minutes to 1 hour after the injection a chill occurs, lasting 20-30 minutes. This is followed by a rapid rise of temperature which later falls abruptly, and then tends to remain remittent in favorable cases.

2. *Leukocytes.*—Usually there is an early leukopenia within 4 hours, due mainly to a disappearance of polymorphonuclears. Within about 12 hours this is followed by a moderate polymorphonuclear leukocytosis.

3. *Blood pressure* is usually decreased by 10-15 mm. after the chill.

4. *Coagulation Time.*—Only slight and insignificant changes have been observed.

5. *Antibodies.*—No essential difference from the specific response to the injection of typhoid antigen in a normal individual was observed in these typhoid patients. It is to be noted, however, that conditions here differ somewhat from those in which an heterologous protein is injected.

The reaction in arthritis cases agrees essentially in the first four particulars with the reaction in typhoid fever. The antibody response will be considered later. Most clinicians seem agreed in regarding the severe chill as the most important factor in this reaction. It is evident that a chill alone cannot be responsible for the marked relief from pain and for the remission in the course of an acute arthritis. It is possible, however, that the resulting hyperpyrexia may have an effect in favoring immune reactions and antibody production.

[9] Miller: Jour. Am. Med. Assn., 1917, 69, p. 765.

Rolly and Meltzer,[10] in a study on the effect of high temperatures, injected a series of rabbits with small doses of typhoid bacilli or cholera vibrios. Some of these rabbits were kept in an oven so that their body temperature remained between 40 and 42 C. The controls were kept at ordinary room temperature. They observed a higher average production of agglutinins and lysins in the high temperature rabbits than in the controls. They also showed that phagocytosis by human leukocytes in vitro occurs at its optimum between 37 and 40 C.

More recently, Winslow, Miller and Noble[11] investigated the effect of moderately high temperatures on the production of lysins against sheep blood corpuscles. They kept their rabbits in an oven at a moderately high temperature, 29 to 32 C. Injections of sheep cells were given and the hemolytic titer estimated each week. They found that the rate of lysin production in the heated rabbits was slower than that in the controls.

These experiments may indicate that a moderately high temperature lowers the general vital resistance to a beginning infection. Once an infection is established, however, according to Rolly and Meltzer's results, hyperpyrexia may bring about a greater production of antibodies.

If a specific leukocytosis could be observed, one might think that this would be a factor in overcoming the infection, but the degree of leukocytosis seems only to parallel the severity in each case.[12] Gay and Claypole[13] believed that a specific hyperleukocytosis occurred in typhoid immune rabbits, following injections of typhoid bacilli, but McWilliams[14] later showed that both normal and immune rabbits respond to about the same degree. The leukocytosis therefore cannot be regarded as specific.

The slight variations in blood pressure and coagulation time are evidently insignificant.

We pass now to a consideration of the antibody response. Very little work on this aspect of the question has been done, yet it seems as if the explanation of a therapeutic response, in the case of any infection, to the injection of a substance not specifically bactericidal to the infecting organism, should logically be sought in the liberation of specific antibodies against that organism. Ordinarily when the body overcomes a streptococcic infection, for example, we believe this is accomplished by the production of antibodies against the streptococcus. Why, then, should we not suspect that the therapeutic effect of foreign protein injections in infections, obviously not due to the foreign protein per se, has as its basis the liberation of specific antibodies against the causative organism? In the case of arthritis we believe that the symptoms persist because the antibodies which should normally be produced are for some reason not liberated.

In the light of Ehrlich's receptor theory, we recognize three distinct phases in the production of antibodies: (1) the sensitization of the

[10] Deutsch. Arch. f. klin. Med., 1908, 94, p. 335.
[11] Proc. Soc. Exper. Biol. and Med., 1916, 13, p. 93.
[12] Cross: Journal-Lancet, 1917, 37, p. 764.
[13] Arch. Int. Med., 1914, 14, p. 662.
[14] Jour. Immunol., 1916, I, p. 159.

tissue cells; (2) an overproduction of sessile receptors; (3) the liberation of these receptors from the cells. A perfect antigen is one which causes all of these phases to be completed. It seems a plausible hypothesis that in those cases of arthritis, etc., which show a rapid therapeutic response to injections of foreign protein, the etiologic organism is acting only as an imperfect antigen. It sensitizes the cells and causes sessile receptor production, but does not give the necessary stimulus to cause these receptors to be thrown off. The foreign protein then supplies this stimulus, the system is flooded with specific antibodies, and a remission of the infection results. Although a variety of proteins may serve to complete this second phase, the reaction is still absolutely specific, since there must be a specific stimulation of cells, and a final liberation of specific antibodies.

In this connection I would recall the experiments of Bruck.[15] According to Ehrlich's theory a molecule of tetanus toxin is composed of a haptophore group, which unites with the cell receptor, and a toxophore group, which is responsible for the poisoning of the cell. This toxophore group can be destroyed by various methods such as heat, light, and long preservation. The toxin has then become a toxoid. The haptophore group can still unite with a sessile cell receptor, or with a specific antibody, but because of the absence of the toxophore group the molecule has become innocuous. Bruck injected rabbits with small repeated doses of such a toxoid. The rabbits were found to produce very little, or no free antitoxin, the amount being dependent on the amount of effective toxophore groups which still remained in the toxoid. He further showed that rabbits injected with tetanus toxoid, during the first few succeeding hours possessed a greater tolerance for tetanus toxin, but that later they showed an actual hypersensitiveness, being killed by only a fractional portion of the ordinary lethal dose of toxin.

These results Bruck interpreted as follows: The haptophore group of the toxoid at first is anchored to the specific cell receptor. The toxin molecule introduced soon afterward, therefore, cannot reach the cell, since all the receptors are blocked. Later, however, an overproduction of sessile receptors occurs, and a hypersensitiveness to toxin results, since the avenues of approach to the cell are increased many times. The toxoid has acted as an inferior antigen. It has brought about the first and second steps of antibody production, namely, (1) sensitization of cell receptors, and (2) the overproduction of similar sessile receptors, but it has lacked the necessary stimulus to cause the discharge of these receptors. Had the rabbits been immunized with small doses of unchanged toxin this third stage would have been completed. Bruck therefore sees in the toxophore group the necessary stimulus for the setting free of the receptors. Might not a foreign protein, in an analogous way, supply such a stimulus? It was with this hypothesis in view that I undertook an investigation of the effect of foreign protein on antibody production. The experiments have been conducted entirely with rabbits, because all the factors can thus be much more accurately controlled than in a purely clinical investigation.

[15] Ztschr. f. Hyg. u. Infections-Krankh., 1904, 46, p. 176; 48, p. 113.

Several other investigations have been reported which touch on the immune reactions in foreign protein therapy.

Jobling and Petersen[16] report a "mobilization of ferments," notably protease and lipase, following foreign protein injections. Later[17] they point out an "increase in antiferment," dependent on "highly dispersed, unsaturated lipoids," and conclude that "the main therapeutic factor is probably an increased dispersion of colloids."

Culver[18] investigated the opsonin and lysin content in the serum of patients with gonorrheal arthritis, after intravenous injections of gonococcic vaccine and proteose. He reports a rise in titer of these antibodies.

Bull[19] reports a rise in the antibodies of typhoid immune rabbits after the injection of typhoid bacilli; but his results could not be confirmed by Teague and McWilliams,[20] who suggest a new theory to explain the therapeutic effect on cases of typhoid, of intravenous injections of typhoid vaccine. On the supposition that blood serum is more bactericidal than lymph, they base the theory that the vaccine causes a greater flow of bactericidal serum into the lymphoid organs, and thus overcomes the local infection. It does not appear that this theory has been substantiated.

On the basis of the hypothesis that the foreign protein reaction serves as a stimulus to set free specific antibodies, I first investigated opsonins for the streptococus. This organism is notably a poor antigen, in that it is difficult to obtain a high antibody titer against it. An animal sensitized with this organism should therefore be a favorable subject for the liberation of antibodies by foreign protein.

Series 1.—Two rabbits were injected intravenously at 3-day intervals with 1 c c of a fresh suspension of killed Streptococcus viridans. The organism was grown on blood agar slants, and a 24-hour growth washed off with 10 c c salt solution. This emulsion was heated at 60 C. for 1 hour to kill the bacteria, and 1 c c of this vaccine used as a dose. A fresh vaccine was made each time. The fourth injection consisted of ½ c c of living streptococci. Five days later the opsonic index was determined, and 2 c c of human ascitic fluid injected intravenously.

The method of determining the opsonic index was that of Wright, using the experimenter's own leukocytes and serum as a standard.

Both rabbits were found to have a very low opsonic index 5 days after the fourth injection of streptococci; 2 c c of human ascitic fluid were then injected. and a marked rapid rise of opsonins occurred, the total increase being 700% in 48 hours. Apparently the foreign protein acted as an effective stimulus to liberate sessile receptors.

[16] Jour. Am. Med. Assn., 1915, 65, p. 515.
[17] Ibid., 1916, 66, p. 1753.
[18] Jour. Lab. and Clin. Med., 1917, 3, p. 11.
[19] Jour. Exper. Med., 1916, 23, p. 419.
[20] Jour. Immunol., 1917, 2, p. 185.

After 18 days the opsonic content was again found to be at a low level. Two further injections of streptococci were then given. This time an interesting variation in response occurred. The opsonic index of Rabbit 17 rose, while that of Rabbit 311 continued to fall. In the former the streptococci evidently acted as a perfect antigen, causing all three stages of antibody production, while in the latter the final liberation of antibodies did not take

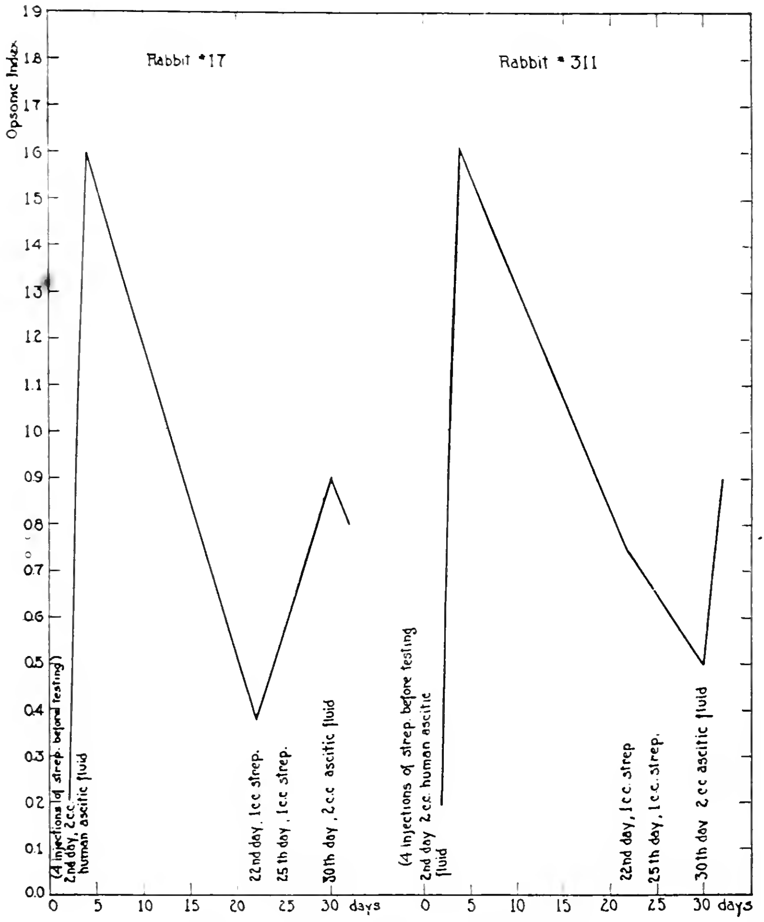


Chart 1 (Series 1).—Opsonins for streptococcus.

place. This supposition was confirmed by the effect of an injection of ascitic fluid. In Rabbit 17 there resulted no further increase in opsonins. The stimulus of the antigen had been sufficient to liberate all antibodies. In Rabbit 311 a rapid liberation of opsonins followed the foreign protein injection. In this case we picture the tissue cells as loaded with an overproduction of

specific sessile receptors. The foreign protein reaction then acted as an effective stimulus to cause the cells to throw off these receptors.

Series 2.—In this series 2 rabbits were injected with fresh suspensions of meningococci, and human serum was used as foreign protein. Rabbit 395

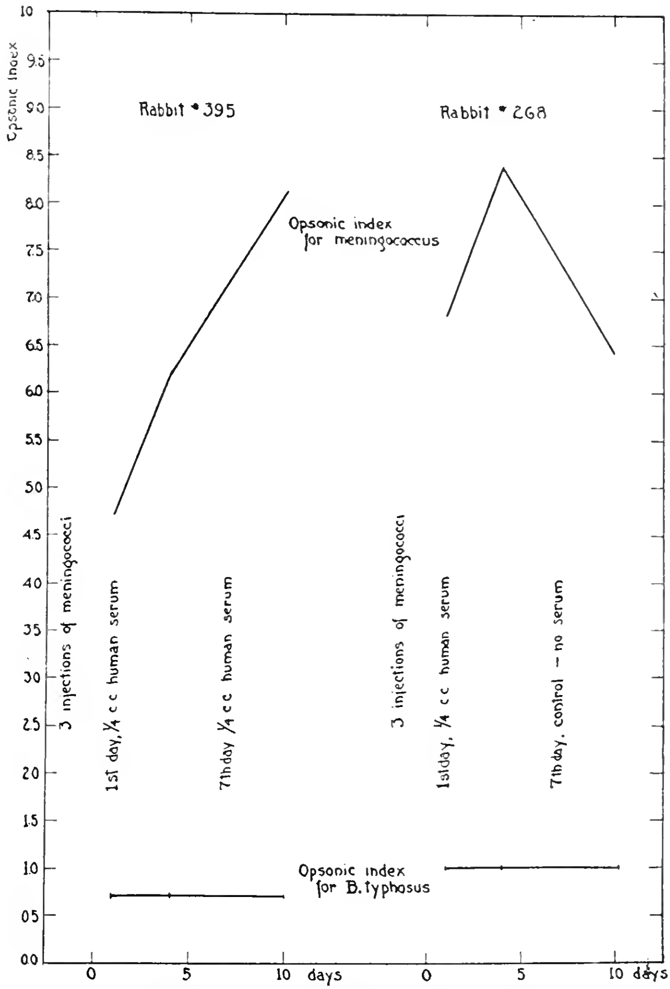


Chart 2 (Series 2).—Opsonins for meningococcus.

received 2 injections of serum with a resulting rise after each injection. Apparently the cells were not completely desensitized by the first serum injection. Rabbit 268 received no second injection and showed a gradual decrease in opsonins.

In order to demonstrate whether these antibodies were specific for meningococci, and also as a control of the technic, the serums were also tested each time for opsonins against B. typhosus. The typhoid opsonic index remained low and unchanged throughout.

Series 3.—Two rabbits were given 4 intravenous injections of S. viridans at 3-day intervals, as in Series 1. Six days after the fourth injection the opsonic index was determined. Rabbit 31 then received 1 c c of human serum intravenously, while Rabbit 32 was used as a control and received no serum. The former showed a rise of 38% on the third day following, while the opsonic index of the latter remained stationary. The control test for B. typhosus remained unchanged in both cases.

During the next 40 days 4 injections of a dextrose broth culture of streptococcus were given. Six days after the last injection the opsonic index was again determined. It was found lowered in both rabbits. Rabbit 32 then received 1 c c of human serum. There followed a rise of nearly 300%. Rabbit 31 showed a spontaneous rise, but to a much less degree. A check opsonic determination for meningococci showed no variation.

It was now thought that if an increase in opsonins occurred, following foreign protein injection, it should also be possible to demonstrate a simultaneous rise in the agglutinin titer for streptococci.

The agglutinin titer was determined by the microscopic method as follows: The rabbits were bled from the ear veins, the blood allowed to clot, and the serum separated by means of rapid centrifuging. Varying dilutions of the serum were then made in 0.9% salt solution. Hanging drop preparations were made by placing on a coverslip, with a small platinum loop, 1 drop each of diluted serum and of a 15-hour dextrose-broth culture of streptococcus. The coverslips were then inverted on vaselined hollow ground slides, and left at room temperature. Readings were then taken under the microscope at the end of 3 hours. Positive agglutination could be observed in the lower dilution, consisting of definite clumping of the streptococcal chains. The highest degree of dilution in which a positive test occurred was recorded as the agglutinin titer.

The first tests were made simultaneously with the last opsonic determinations. In response to the first serum injection Rabbit 32 showed a rise in agglutinin titer of 250%. Rabbit 31, which served as a control at this time, also showed a slight rise (50%) and continued to rise gradually. The agglutinin titer of Rabbit 32 began to fall after the third day. All the sessile receptors apparently had been freed by the foreign protein injection, while in Rabbit 31, without the stimulus of foreign protein, they were liberated to a less degree and more slowly.

In order to resensitize the tissue cells 2 c c of broth culture were now injected. Seven days later the agglutinin titer had fallen very low in both rabbits. This time Rabbit 31 received 1 c c of human serum, while Rabbit 32 served as control. A rapid rise of agglutinin titer occurred in the former which then remained stationary. In the control rabbit a more gradual rise occurred. Thus the specific response to the foreign protein injection was again observed.

Series 4.—It now became necessary to investigate whether or not foreign protein injections would serve to bring about a mobilization of antibodies against an organism which was known to be a good antigen, that is, one which when injected into an animal, would readily cause the production of a high titer of free antibodies. The typhoid bacillus is known to be such a perfect antigen, when injected into rabbits. Indeed, it is not unusual to obtain an agglutinin titer against typhoid as high as 1:500,000 dilution.

Two rabbits were injected repeatedly with emulsions of typhoid bacilli, until they showed an agglutinin titer of 1:50, on testing by the macroscopic method; 1 c c of guinea-pig serum was then injected and the agglutinin titer again determined in 48 and 72 hours. No rise in titer was apparent.

Further injections of typhoid bacilli were then given until the agglutinin titer reached 1:100. Then ½ c c of human serum was injected. Again no

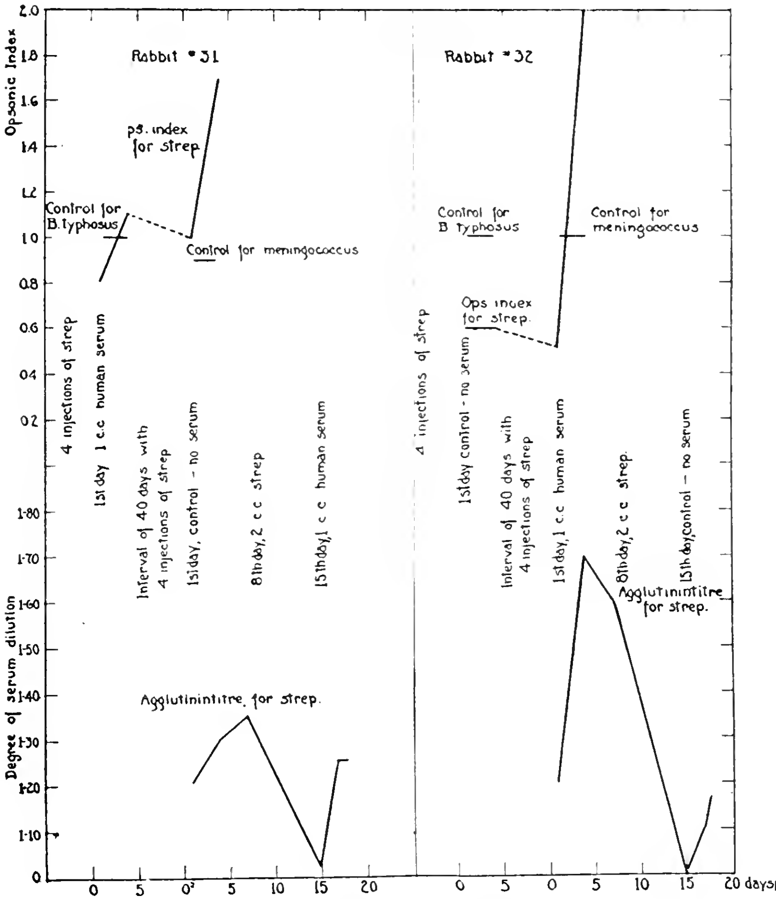


Chart 3 (Series 3).—Opsonins and agglutinins for streptococcus.

increase in agglutinins could be observed during the next 72 hours. A second injection of human serum did not change the titer.

In order to check these negative results 2 more rabbits were immunized with typhoid bacilli. After 2 injections of bacilli the agglutinin titer was found to be zero, by the microscopic hanging drop method, as described under Series 3; ½ c c of human serum was then injected, and the antibody titer again determined on the third day following. Again no agglutinins were found. Evidently the rabbits had not yet been sufficiently sensitized against the bacilli.

Immunization with typhoid bacilli was then continued until the microscopic method revealed an agglutinin titer of 1 : 3,000. Two c c of human ascitic fluid were then injected intravenously. No rise in agglutinin content resulted.

The serum of these rabbits was also tested for complement fixing bodies, with the idea in mind that the foreign protein reaction might possibly bring about a liberation of such antibodies, although it apparently had no effect on the agglutinin content.

As an antigen in the complement fixation test an old suspension of typhoid bacilli in salt solution, to which 0.3% tricresol had been added, was used. The test was carried out with the usual technic, using this typhoid antigen, washed sheep blood cells, fresh guinea-pig complement, and rabbit amboceptor. The serum of the typhoid immune rabbits was inactivated by heating at 56 C. for ½ hour, then diluted, and the fixing power of a given quantity tested. It was found that the ability of the inactivated serum to fix complement was not changed in the slightest degree by foreign protein injections.

We must conclude that foreign protein injections have no effect on the antibody production in typhoid immune rabbits. The typhoid bacillus is apparently a perfect antigen in rabbits, and no foreign protein is needed to stimulate the liberation of antibodies against it.

How, then, can we reconcile this with the apparent therapeutic benefits observed in some human patients? May it not be possible that some patients do not react completely to the antigenic stimulation of the typhoid bacillus? In such cases the foreign protein might well serve as a stimulus causing the sensitized cells to throw off their sessile receptors. This sudden flooding of the system with antibodies might then be responsible for the remission in the disease. This hypothesis seems to be supported by the succeeding series.

Series 5.—Thus far I have recorded observations on opsonins and agglutinins. It seemed desirable also to investigate the response of a third type of antibodies, lysins, to foreign protein injections. Since it is difficult to make accurate determinations of the lysin content of a serum for a specific bacterial cell, we decided to use as our antigen washed sheep corpuscles. A hemolytic titer can be very accurately obtained, while a bacteriolytic titer, obtained by the method of extinction and plating, is modified by too many possible errors of technic.

It is well known that rabbits will readily produce lysins against sheep corpuscles. Common use is made of this fact in producing amboceptor for the hemolytic system of the Wassermann reaction. We may say, therefore, that sheep cells act as a good antigen when injected into rabbits. This would allow us to predict, on the basis of the preceding series, that foreign protein injection into rabbits sensitized against sheep cells would have little effect on the liberation of specific lysins. It has, however, been frequently observed in this laboratory that a great variation exists in the readiness with which different rabbits will produce amboceptor when injected with sheep cells. Rubinstein[21] recently studied the hemolytic titer of rabbits after 3 injections of sheep red cells. The titer was determined before each injection, and 7 days after the third. He found that of 11 rabbits only 8 showed a definite marked rise in lysins after each injection. In one of the remaining 3 the titer remained the same, and in the other 2 there was even a decrease in lysin content after successive injections. In such rabbits as the latter 3 the sheep cells evidently acted only as an inferior antigen, and in such cases we would expect, on the basis of our previous work, to be able to liberate antibodies by means of foreign protein injections.

[21] Campt. rend. Soc. de biol., 1917, 80, p. 908.

The hemolytic system in these experiments consisted of 0.10 c c fresh guinea-pig complement (diluted 1:10), 0.5 c c of a 2% suspension of fresh washed sheep corpuscles, 1.5 c c of 0.9% salt solution, and graduated amounts of inactivated rabbit serum. The least amount of serum that would cause complete hemolysis after incubation at 37 C. for 1 hour was recorded as the lysin titer.

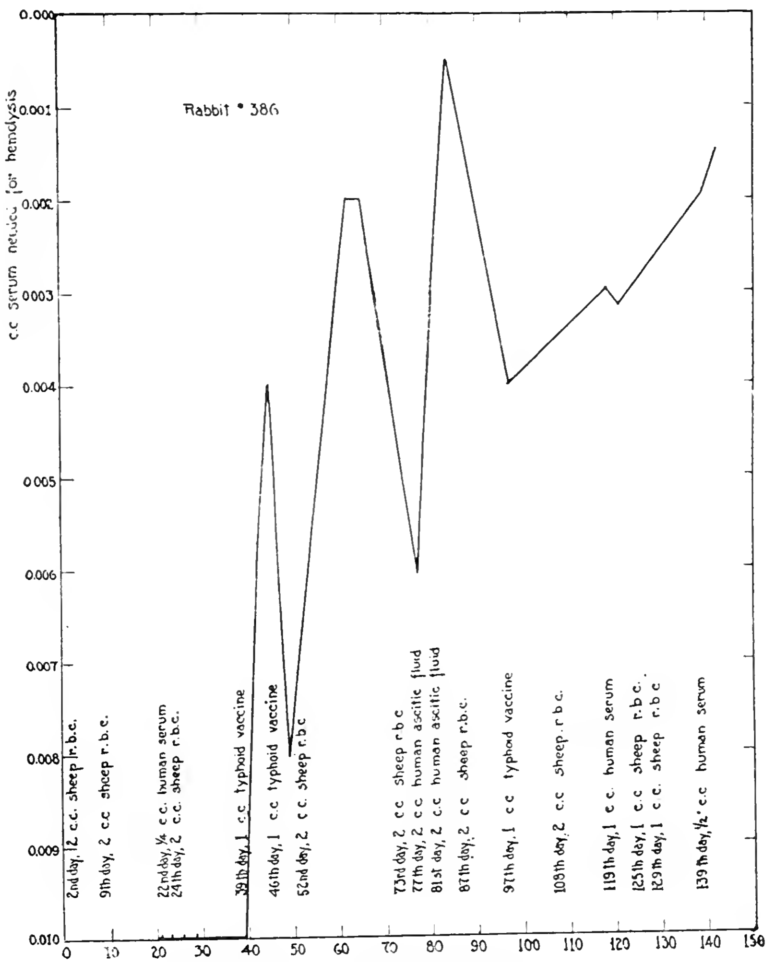


Chart 4 (Series 5).—Lysins for sheep cells.

A total of 20 rabbits was used in this series. The rabbits were injected intravenously with 2 initial doses of sheep corpuscles in salt solution. As a rule the lysin content was then measured, 10-12 days after the last injection, it having been determined that the lysin titer normally reaches its height by the end of this period. After foreign protein injections, either human serum

or typhoid vaccine, the lysin content was again determined, usually in 72 hours. in order to give ample time for any resulting change in titer to take place.

In Rabbit 386 sheep cells evidently acted as an inferior antigen, the lysin content 11 days after the second injection being very low and remaining so; 0.25 c c of human serum injected on the 17th day after the last injection did not influence the lysin content. Believing that this was due to the fact that the tissue cells were no longer properly sensitized, another injection of sheep

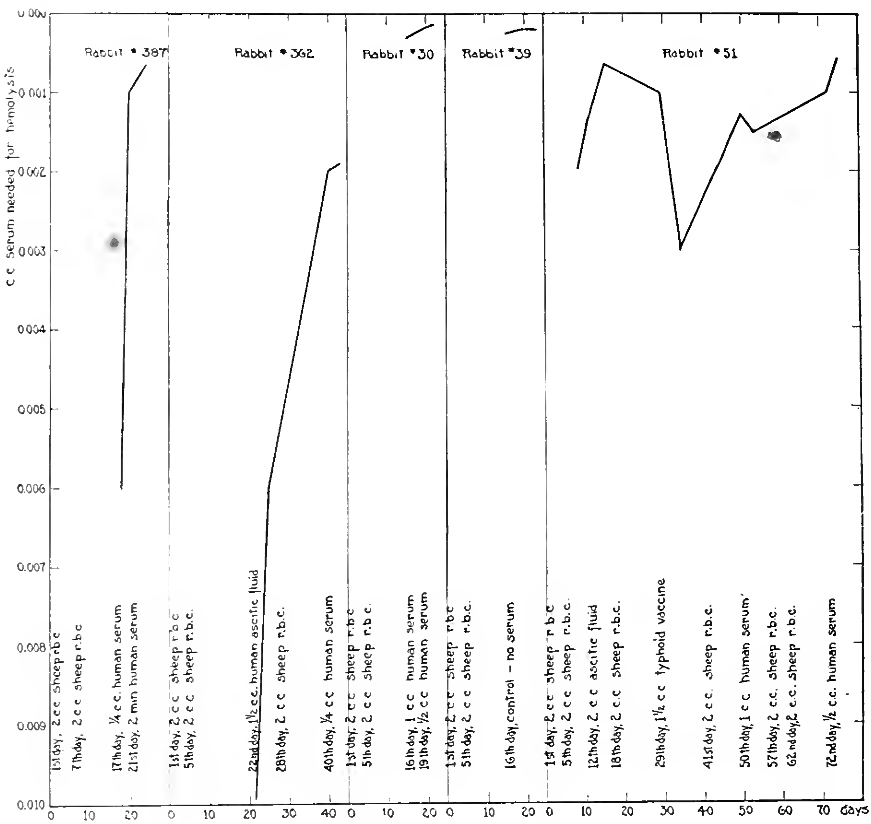


Chart 5 (Series 5).—Lysins for sheep cells.

cells was given. Eleven days after this the titer was still at the same low level. An injection of foreign protein (typhoid vaccine) was then given, and an immediate marked liberation of lysins occurred. These antibodies disappeared rapidly. A second injection of typhoid vaccine in 7 days had no effect on raising the lysin titer, but the curve continued to fall rapidly. A third injection of red cells was then given and this time acted as a perfect antigen. The lysin content rose to its height in 10 days and remained high for several days. After it began to decrease another injection of red cells was given, but this time an injection of ascitic fluid was given only 4 days

later, in order to determine whether an early stimulation by foreign protein would serve to liberate antibodies. A very pronounced mobilization of lysins followed.

This alternating introduction of red cells and foreign protein was continued. Every time the mere injection of antigen (red cells) failed to produce lysins in the rabbit serum a mobilization of these antibodies could be effected by foreign protein. When, on the other hand, there was a normal response to the antigen, the foreign protein was followed by no further liberation of lysins. After the last 2 injections of red cells apparently all the receptors were not spontaneously liberated because there was a slight response to foreign protein 10 days later.

Rabbits 387 and 362 were of the same type, as they failed to respond to injections of red cells by a proper production of antibodies, but when then injected with foreign protein a marked liberation of lysins occurred.

Rabbits 30 and 39 responded very well to the antigenic stimulation of sheep cells; Rabbit 39 was used as a control while Rabbit 30 received human serum on the 11th day following the last injection of antigen. No marked difference was noted in the reactions of the 2 rabbits. This was observed also in a number of other cases. When the titer is already high, foreign protein will not bring about a further rise in antibody content.

Rabbit 51 represents a negative result which cannot be readily explained. The typhoid vaccine injected on the 29th day should have been followed by a rise in antibodies, according to our other observations, since the red cells 11 days earlier had not produced a liberation of lysins. In other respects, however, this curve is in accord with the general results.

SUMMARY

In rabbits sensitized with streptococci a definite liberation of specific opsonins and agglutinins follows the injection of foreign protein.

A similar rise in specific opsonins also occurs in rabbits sensitized with meningococci.

Foreign protein injections have no effect on antibodies in typhoid immune rabbits.

In suitable rabbits, which do not readily produce lysins against sheep corpuscles, the injection of foreign protein within 10 days after the injection of antigen is followed by a marked liberation of specific lysins.

A variety of foreign proteins can be used. Human serum, typhoid vaccine, human ascitic fluid, and guinea-pig serum proved equally efficacious.

CONCLUSION

The intravenous injection of foreign protein serves as a stimulus for the liberation of specific antibodies in animals in which the previously injected antigen is unable to cause such a liberation. This insufficiency may lie either in the antigen or in the rabbit.

OBSERVATIONS ON THE PRESENCE OF THE MENINGOCOCCUS IN THE BLOOD

Kenneth F. Maxcy
Captain, M. C., U. S. Army

With the growth of our knowledge of epidemic meningitis, the presence of the causative organism in the blood stream has received increasing attention. From an essentially meningitic affection, the disease has come to be viewed by many as a primary sepsis with secondary invasion of the meninges as the usual — but not the necessary — accompaniment. Strong evidence in support of this view has been brought recently by the work of Baeslack[1] and Herrick[2] and their co-workers. The following observations, made at Camp Beauregard during the epidemic in Jan. and Feb., 1918, are of interest in this connection.

It was considered of practical importance in the study of the epidemiology of the disease at this camp to ascertain whether the abortive type of case was of common occurrence. The carrier theory was not altogether satisfactory in tracing the development of the epidemic. The term "abortive type" is used in this relation for those cases of meningococcus infection in which the organism invades the blood stream for a short time but is overcome by natural immunity before secure localization in the meninges is effected. Such cases might easily go unrecognized — passing as "grippe" or "influenza"— and if of frequent occurrence might play an important rôle in the spread of the disease.

At the suggestion of Major George Draper, efforts were made to determine this point. This entailed close watch over the admissions to the base hospital and daily visits to the regimental infirmaries to discover cases that might fall into such a group. Particular attention was paid to patients who were thought to have influenza, or regarded as "meningitis suspects" or "meningitis contacts." An examination was made for evidence suggesting a meningococcus infection, that is, petechial eruption, fever, malaise, headache, etc., without an obvious

Received for publication Sept. 2, 1918.

[1] Jour. Am. Med. Assn., 1918, 70, p. 684.
[2] Arch. Int. Med., 1918, 21, p. 541.

explanation. If the clinical picture was thought suggestive, a blood culture was made.

TECHNIC

The technic was similar to that advocated by Baeslack,[1] namely, 10-20 c c of blood were withdrawn and introduced into a flask (or small grape-juice bottle) containing 100 c c of a 1 per cent. glucose, salt-free broth which was usually enriched by adding a small amount of sheep serum water. Special care was taken to adjust the reaction of the medium so that it was neutral or just slightly acid after autoclaving. At first the titration was carried out with phenolsulphonephthalein as an indicator and the first faint trace of pink, permanent after boiling, as the neutral point. This was found unsatisfactory, and later use was made of solutions of phosphate mixtures of known hydrogen ion concentration as standards, to determine the hydrogen ion concentration of the medium more exactly with cresol purple as an indicator. An especial effort was made to keep the flask approximately at body temperature from the time the blood was withdrawn until it had been placed in the incubator. For this purpose, a fireless cooker heated by hot water bottles was used, when the cultures were made at a distance from the base hospital.

At the end of 48 hours and again at the end of 72, 2 c c of the culture were pipetted off from just above the blood, which had settled out in the bottom of the flash, and poured over the surface of laked sheep blood dextrose agar plates. After being allowed to stand for an hour the plates were tilted to drain off the moisture. They were examined in 18-24 hours for the characteristic colonies. The original flask was kept for 5 days, and if it showed no suspicious turbidity, and the blood-agar plates had proved negative, the culture was declared sterile.

RESULTS

Of a large number of patients examined, 27 were selected as clinically suggestive of possible meningococcus infection. None of these showed petechiae. All fell in to the group of fever, malaise, headache, etc., of unexplained origin, many of them being persons who had been in close contact with actual cases of epidemic meningitis.

In these 27 cases blood cultures were made. The results were entirely negative. In no case could the meningococcus be demonstrated.

As a result of this work, it was inferred that the "abortive type" of meningococcus infection, as defined above, was not of frequent occurrence, if, indeed, it occurred at all in the epidemic at Camp Beauregard. It certainly could not be considered a factor in the spread of the disease.

During the course of this investigation two cases of epidemic meningitis were encountered which illustrate very beautifully how brief and relatively unimportant the blood stream invasion may be in the one, as contrasted with the paramount importance which it may assume in the other, certainly more rare, type of case. Facts bearing on this point are of so much practical importance in determining the relative

value of the intraspinal and the intravenous mode of administering antimeningococcus serum that it is thought worth while to report the histories.

CASE 1.—The patient was admitted to his regimental infirmary late in the afternoon of Feb. 13 with headache, malaise and fever. The following morning about 10 o'clock when he was examined he had no meningeal symptoms and no petechiae, and might well have passed as a case of "grippe." A blood culture was made at this time, and proved positive for the meningococcus.

Subsequently the symptoms became exaggerated, and he began to vomit and grow dull mentally. He was transported to the base hospital as a meningitis suspect. By 2 o'clock of the same day his meningeal symptoms had become pronounced and a few petechiae were visible over the abdomen. A lumbar puncture was made and the fluid obtained was turbid and contained meningococci. Antimeningococcus serum was given intraspinally. One hour later, that is 5 hours after the first and positive blood culture, a second blood culture was taken with exactly the same technic and under more favorable conditions, since the case was now in the base hospital, and it proved to be entirely negative for the meningococcus.

The meningococcus obtained from the first blood culture and from the spinal fluid proved to be a normal strain. It agglutinated slightly up to a dilution of 1:100 with para-normal serum, but very well up to a dilution of 1:400 with the normal and polyvalent serum.

The further history of this case was as follows: He was given 3 intravenous treatments on successive days with intensive intraspinal treatment over a longer period. He responded well and on the 5th day he was greatly improved, temperature, pulse, and respiration had reached normal, and the spinal fluid was clear. On the following day he suddenly became comatose with stertorous breathing and died in a few hours. At necropsy the meninges were found to be entirely clear of exudate, but there was a large hemorrhage over the base of the brain apparently originating from the basilar artery or one of its branches.

In this case, there was apparently a transient meningococcus invasion of the blood stream — a meningococcemia. The organism disappeared from the blood stream within 24 hours, localizing in the tissue of predilection — the meninges. Here it was attacked successfully by the intraspinal administration of serum supplemented, perhaps unnecessarily, by intravenous injections. Death occurred from a vascular accident, the exact cause of which was not apparent.

In direct contrast to the foregoing is the second case:

CASE 2.—The patient began to have chilly sensations and feel sick while drilling on the afternoon of Jan. 11. The following morning he reported at sick call and was taken to the regimental infirmary. There he remained 4 days, passing as a case of "influenza." On Jan. 15 he was transferred to the base hospital because he did not improve. At this time he did not seem particularly sick, but because he vomited once or twice and had just a suggestion of a stiff neck, a lumbar puncture was made and a slightly turbid fluid obtained. The patient was immediately transferred to the meningitis ward where specific treatment was instituted by both the intravenous and intraspinal routes. The

details of his treatment are shown in Table 1. The spinal fluid quickly became clear, but the fever persisted and there was nausea, headache, vague bodily pains, restlessness and general weakness. He was given a second series of intraspinal treatments with slight temporary improvement. On Feb. 8, 3 weeks after the onset of his illness, a lumbar puncture again showed the spinal fluid clear, not increased in pressure, and free from organisms—although the same clinical symptoms persisted. The coexistence of another disease was suspected, but no proof could be brought by physical examination or laboratory aids.

TABLE 1

DETAILS OF TREATMENT FOLLOWED IN CASE 2

Date	Maximum Temperature	Intraspinal Serum, c c	Intravenous Serum, c c	Remarks
Jan. 17	101.8	25	..	Fluid cloudy; organisms not seen, but grown in culture
18	102.8	30 30	60	
19	101.6	30	60	
20	103.4	30	60	
21	103.8	—	..	Fluid clear; no organisms; few pus cells
22	99.0	30		
30	101.3	30	..	Fluid clear; few pus cells
31	100.8	—		
Feb. 1	98.0	30		
2	96.6	—		
3	98.0	30	..	Fluid clear; slight coagulum
8	101.6	—	..	Fluid clear; few cells; 5% mononuclears; leukocytes, 20,400
14	103.2	..	..	Leukocytes, 17,000; 77% polymorphonuclears
19	98.0	..	..	Blood culture No. 1, positive
21	100.8	..	..	Blood culture No. 2, positive; patient desensitized to serum
22	102.8	..	10	
23	102.4	..	40	
23	102.4	..	40	Leukocytes, 18,000
24	104.2	..	60	Blood culture No. 3, negative
25	104.6	..	40	Blood culture No. 4, positive
26	99.8	..	60	
27	104.0	..	—	
28	105.4	..	60	Blood culture No. 5, negative
Mch. 1	104.6	..	60	Leukocytes, 16,800
2	102.2	..	60	
3	102.2	..	60	
9	99.6	..	..	Blood culture No. 6, negative
15	100.9	..	..	Blood culture No. 7, negative
17	97.8	..	..	Leukocytes, 8,600

On Feb. 17, 4 weeks after the onset of his illness, it was noted that the patient had an eruption. It consisted of red macules or maculopapules, varying in size from a pea to a dime, some of them definitely nodular. They were distributed chiefly over the extremities, especially over the dorsum of the hands and feet with a few over the chest. They did not disappear on blanching the area; did not itch and were not painful. The eruption immediately suggested a hematogenous infection of some description, the nodules being somewhat similar to those seen in staphylococcus septicemia.

On Feb. 19 a blood culture was made and the meningococcus recovered. Two days later this was confirmed by a second positive culture. After the patient had been desensitized to horse serum, the intravenous injection of antimeningococcus serum was begun. The dosage and intervals are shown in the table. In all 490 c c were given in a period of 10 days. The patient had violent reactions after the first 4 injections of this series in spite of his desensitizing

doses, but thereafter had very little trouble. After the sixth injection the blood culture became negative and remained so. The patient improved steadily and the temperature gradually declined until it reached normal. April 17 he was apparently well.

The meningococcus recovered in the blood cultures was of the normal type. It agglutinated up to 1:400 with normal and polyvalent serums. With the patient's own serum, taken before treatment was instituted on Feb. 21, it agglutinated up to 1:20. With a specimen taken after 4 intravenous treatments it agglutinated up to 1:40, showing some increase in the agglutinin content of the blood, presumably as a result of the intravenous injections.

In this second case, then, we were dealing with a true meningococcus sepsis. The meningitis was transitory and yielded quickly to intraspinal treatment. The organism, however, was able to survive and perhaps to multiply in the blood stream. No focus of distribution could be demonstrated. Nasopharyngeal swabs were repeatedly negative. There was never any indication of chronic infection in the upper respiratory tract, and the heart was entirely normal. The patient recovered fully after persistent intravenous injection of antimeningococcus serum.

Such cases of meningococcus sepsis as this are probably quite rare. That a transient meningococcemia is far more frequent is indicated by the fact that positive blood cultures are seldom obtained late in the disease, and by the technic of blood culture itself, namely, the necessity of using large quantities of blood in a large amount of broth. Indeed, in the light of our present knowledge, it must be admitted that there may be many cases in which the organism never gains a secure foothold in the blood stream, but localizes directly in the meninges. The question of the mode of invasion from the posterior nasopharynx is still an open one. Each case should be considered a problem in itself and the treatment directed as the particular needs of that case indicate.

CONCLUSIONS

During the epidemic at Camp Beauregard in February, 1918, 27 blood cultures done on patients suspected of being cases of abortive meningococcus infection were negative.

In this epidemic the abortive meningococcus infection was not a considerable factor in the epidemiology of the disease.

A case of meningococcus infection is reported in which the blood stream invasion was transient and the organism quickly localized in the meninges where it yielded to the intraspinal injection of serum.

A case of meningococcus septicemia with transient meningeal involvement is reported with recovery only after the injection of large amounts of antimeningococcus serum intravenously.

ACUTE RESPIRATORY DISEASES AMONG TROOPS WITH ESPECIAL REFERENCE TO EMPYEMA

Lynn S. Beals, Benjamin F. Zimmerman,
and
Searle B. Marlow
Captains, M. C., U. S. Army
From the Base Hospital, Camp Custer, Mich.

This study of acute respiratory diseases among troops details the incidence of various respiratory conditions, measles and rubella in their relation to each other and to the more serious conditions, pneumonia and empyema. Out of a total of 9,691 admissions to a base hospital, 4,443 were cases of acute respiratory infections and are the ones included in this analysis. Table 1 and Chart 1 show how these infections appeared in waves, sudden both as to number and severity. During a period of about three months (from Sept. 25 to Dec. 16, 1917) there were 333 cases of measles (including rubella) only three cases having complications—otitis media in each instance, one case requiring a mastoid operation. This period was relatively free from respiratory infections, but when in January the infections of the respiratory tract were very prevalent and severe, not only did there occur many cases of pneumonia and empyema but the cases of measles developed respiratory infections of a severe type, usually of streptococcus origin (See Table 1). The number of cases of measles and rubella did not increase materially but the complications, often serious or fatal, increased greatly, there being 14 cases of empyema, with 9 deaths, and 4 cases of pneumonia. Of a total of 58 consecutive cases of measles about this time the complications were as follows: empyema, 5; pneumonia, 2; tonsillitis, 3; otitis media, 10; and severe bronchitis, 2.

In cases of measles developing empyema it has been definitely and repeatedly observed that the lungs did not at any time become free from râles and that the initial or complicating severe infection of the throat did not show much improvement prior to the onset of the empyema.

The deep red injection of the mucous membrane in the fatal cases continued with the empyema to the necropsy table where the tissues

Received for publication Sept. 13, 1918.

were frequently found to be stained from laked blood; the skin and conjunctiva during life in the severe cases frequently showed marked jaundice of hematogenous origin. In these cases S. hemolyticus was usually demonstrable.

It is noted also that the cases which had a slight but constant fever following the active stage of measles were the ones most liable to have the serious complications of empyema. One reason for this has been shown at necropsy where an interlobar lesion in such a stage of organization has been found as to definitely place its time of onset prior to the signs of empyema.

The most severe chest condition during the first 6 months of 1918 was empyema. Its relation to other diseases and its mortality from

TABLE 1

Total Number of Admissions of Measles and Respiratory Cases (by Months) Sept. 25, 1917, to June 1, 1918

	Total Number of Cases										
	Tonsillitis (A)	Bronchitis (B)	Pharyngitis (C)	Laryngitis (D)	Stomatitis (E)	Rhinitis (F)	Total of A, B, C, D, E and F	Measles and Rubella	Pneumonia and Empyema Col. 17	Total Measles and Respiratory Cases	All Cases in Hospital
Sept.	7	19	......	......	......	6	32	6	2	40	172
Oct.	15	32	9	2	......	3	61	24	5	90	459
Nov.	55	32	13	4	......	3	107	165	5	277	599
Dec.	72	56	18	9	1	7	163	217	14	394	813
Jan.	288	304	117	26	......	4	739	244	63	1,046	1,809
Feb.	296	135	50	9	1	13	504	66	29	599	1,549
March	171	264	110	7	......	8	560	20	54	634	1,288
April	194	317	175	2	......	13	701	58	109	868	1,556
May	92	227	48	1	1	10	379	30	86	495	1,446
Total	1,190	1,386	540	60	3	67	3,246	830	367	4,443	9,691

various points of view is shown in the tables. In this study a condition is designated empyema which shows an acute nontuberculous collection of purulent exudate in the pleural cavity.

The cases of empyema which gave the highest mortality were those which clinically presented the picture of severe septicemia. In the great majority of cases the exudate was thin, slightly turbid fluid, the smear showing pus cells and streptococci. This exudate often accumulated with great rapidity both in the cases secondary to pneumonia and those of primary origin, especially following measles. One severe case of primary empyema following a severe pharyngitis went, in 16 hours, from the stage of fibrinous exudate (a friction rub over the entire chest) to about 1,500 c c lemon yellow turbid fluid containing pus cells and great numbers of streptococci.

The second group of cases, not quite so fulminant in character, showed at the onset of the empyema, the same turbid fluid. The first group (fulminant) had a very high mortality with any operative procedure. The second group (less fulminant) often went to the thick pus stage several days later, at which time a costectomy was made.

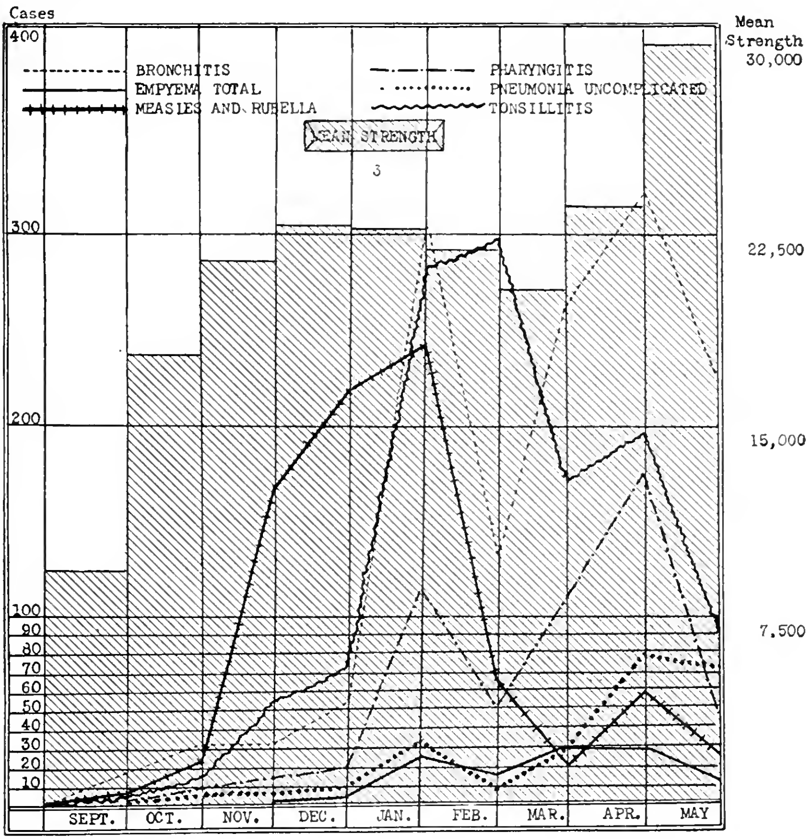
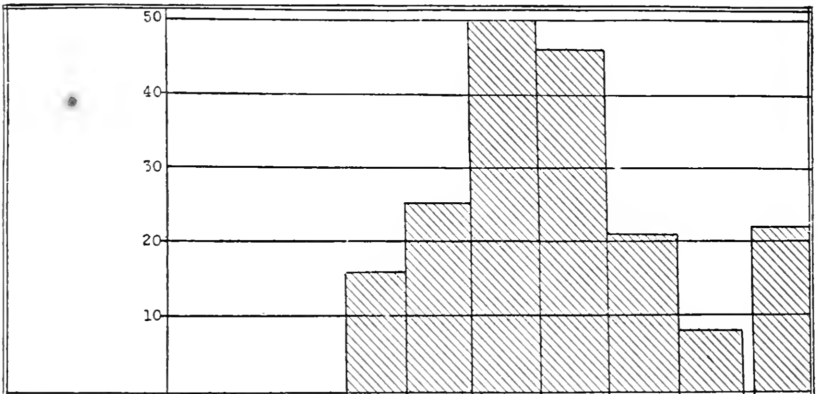


Chart 1.—Admissions of measles and respiratory cases by months.

In a third group of cases the symptoms were not such as to indicate the presence of a serious condition. The signs were of fluid in small or moderate amount. When aspirated after some days these cases showed (1) a turbid fluid containing pus cells and streptococci, or (2) a thick yellowish gray pus with many organisms, usually streptococci. Class 1 of this third group in 2 cases took care of the fluid without any operative interference, the second class did fairly well with costectomy.

A fourth group of three atypical cases which occurred all in one week during the middle of March, were of sudden onset, chill, high fever, small thready rapid pulse, and septic appearance. The chest showed signs of a small amount of fluid. Aspiration showed in all a few c c of very thick yellowish gray pus, one containing a doubtful streptococcus and the other two sterile. These cases had normal temperature in 2 or 3 days and made quick recovery without any operative interference other than the original exploratory tapping.

There seem to be 4 definite stages in the genesis of empyema: fibrinous, serofibrinous, serofibrinopurulent, and purulent.



	SEPT.	OCT.	NOV.	DEC.	JAN.	FEB.	MAR.	APR.	MAY	TOTAL
No. of cases of pneumonia	2	5	5	12	40	18	46	99	81	308
No. of cases of Empyema complicating pneumonia	0	0	0	2	10	9	21	20	6	68

Chart 2.—Relative incidence of empyema complicating pneumonia.

The usual early finding for a variable period of about a week is the serofibrinopurulent exudate. At necropsy there frequently was found a serofibrinopurulent exudate in one pleural cavity, and a fibrinous or serofibrinous in the other. It is important to recognize further, that while the course is usually progressive through the various stages toward the end or purulent stage, the fibrinous or serofibrinous pleuritis may proceed no further than those stages, the case not being a true empyema. These first two stages are frequently found in cases of pneumonia and great care should be exercized that the cases

	Measles			Rubella			Measles and Rubella			Complicated by Pneumonia			Complicated by Pneumonia			Rubella Complicated by Pneumonia		
	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality
September.......	1	....	....	5	....	....	6											
October..........	22	....	....	2	....	....	24											
November........	117	....	....	48	....	....	165											
December........	176	1	0.6	41	1	2.4	217	2	0.9									
January.........	207	9	0.4	37	....	....	244	9	3.8	3	....	....	1	....	....	4		
February........	58	2	3.5	8	....	....	66	2	3.0	2	1	50.0	....	....	....	2	1	50.0
March...........	13	1	7.7	7	....	....	20	1	5.0	2	1	50.0	....	....	....	2	1	50.0
April............	29	2	6.9	29	....	....	58	2	3.4	3	1	33.3	....	....	....	3	1	33.3
May..............	23	3	13.0	7	....	....	30	3	10.0									
Total.........	646	18	2.8	184	1	0.5	830	19	2.3	10	3	30.0	1	....	....	11	3	27.2

[No cases in Sept., Oct., Nov.]	7 Primary Empyema			8 Measles Complicated by Empyema			9 Rubella Complicated by Empyema			10 Total Measles and Rubella Complicated by Empyema			11 Measles Complicated by Pneumonia and Empyema			12 Total Empyema Following Measles and Rubella		
	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality
December........	....	....	....	1	1	100.0	1	1	100.0	2	2	100.0	....	....	....	2	2	100.0
January.........	5	1	20.0	12	7	58.3	....	....	....	12	7	58.3	2	2	100.0	14	9	61.5
February........	4	2	50.0	5	1	20.0	....	....	....	5	1	20.0	....	....	....	5	1	20.0
March...........	6	1	16.7															
April............	5	1	20.0	2	1	50.0	....	....	....	2	1	50.0	....	....	....	2	1	50.0
May..............	2	....	....	1	1	100.0	....	....	....	1	1	100.0	2	2	100.0	3	3	100.0
Total.........	22	5	22.7	21	11	52.4	1	1	100.0	22	12	54.5	4	4	100.0	26	16	61.5

	13 Lobar and Bronchopneumonia Complicated by Empyema			14 Total Empyema			15 Lobar and Bronchopneumonia Uncomplicated			16 All Pneumonias			17 Total Pneumonias and Empyemas of All Kinds		
	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality	No. of Cases	No. of Deaths	% Mortality
September.....	....	....	....	....	....	....	2	1	50.0	2	1	50.0	2	1	50.0
October........	....	....	....	....	....	....	5	....	....	5	...	....	5		
November......	....	....	....	....	....	....	5	....	....	5	....	....	5		
December......	2	....	....	4	2	50.0	10	....	....	12	....	....	14	2	14.2
January.......	10	3	30.0	29	13	44.9	30	5	16.7	46	10	21.8	63	18	28.6
February.......	9	2	22.2	18	5	27.8	9	1	11.1	20	4	20.0	29	7	25.5
March..........	21	11	52.4	27	12	44.4	25	5	20.0	48	17	35.4	54	18	33.3
April...........	20	9	45.0	27	11	40.7	79	10	12.7	102	20	19.6	109	22	20.2
May...........	6	1	16.7	11	4	36.4	75	9	12.0	83	12	11.4	86	13	15.1
Total.......	68	26	38.2	116	47	40.5	240	31	12.9	323	64	19.8	367	81	22.1

* All deaths are recorded by months as of the time entering hospital and not by actual date of death. This table includes all deaths up to June 26, 1918.
Col. 3 is a total of Cols. 1 and 2 and gives the total incidence and mortality, from all complications of all measles and rubella based on Cols. 6, 10 and 11.
Col. 6 is a total of Cols. 4 and 5 and includes those cases of measles and rubella which had pneumonia as to the only complicating chest condition.
Col. 7 includes all cases of empyema which did not follow pneumonia or measles.
Cols. 8 and 9 include (each as designated) those cases of empyema which were primary so far as chest findings were concerned but which followed measles or rubella.
Col. 10 is a total of Cols. 8 and 9.
Col. 11 includes those cases of empyema which followed measles but in which the primary chest condition was definitely a pneumonia with the empyema secondary to the pneumonia.
Col. 12 is a total of Cols. 10 and 11, i. e., all cases which followed measles or rubella.
Col. 13 includes all cases of lobar and bronchopneumonia (uncomplicated by measles or rubella) followed by empyema.
Col. 14 is a total of Cols. 7, 12 and 13 and includes all cases of empyema without regard to any preceding condition.
Col. 15 includes all cases of lobar and bronchopneumonia uncomplicated by empyema or measles.
Col. 16 is a total of Cols. 6, 11, 13 and 15 and includes all cases of pneumonia uncomplicated (15), complicated by empyema (13), complicating measles

of serofibrinous exudate are not thought cases of empyema and operated on. Neither should the possibility of a fluid being purulent be lost sight of.

During the month of January the high mortality of empyema cases was due to those cases which followed measles. These showed great virulence of the infecting organism and a very fulminant course. The high mortality of January was further due to the fact that the course of the new type of empyema was not clearly understood at the outbreak of this condition.

During the month of February, March and April there were only 6 cases of empyema following measles, none during March and only

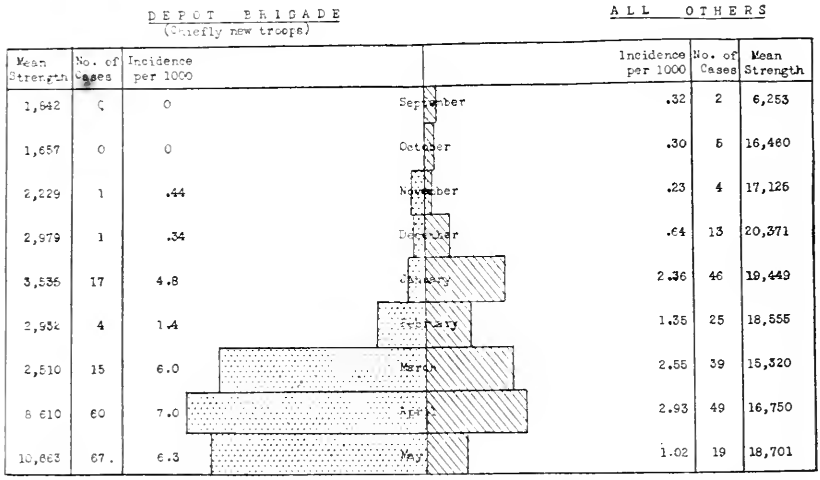


Chart 3.—Incidence of pneumonia and empyema in the depot brigade (chiefly raw recruits) and the other organizations of the cantonment composed of more seasoned soldiers.

2 in April, but there were relatively few cases of measles. During March the mortality from pneumonia complicated by empyema was high (52.4%); nine of the 12 fatal cases for March, however, were admitted to the hospital on or after March 27. Taking a period from March 27 to May 1, 1918, the cases of pneumonia complicated by empyema were 34 with mortality of 50%, the mortality of pneumonia uncomplicated by measles or empyema for the same period was 14.3%. These are more nearly the real figures for this group of cases during this period than those from April 1 to May 1. The mortality of uncomplicated cases of pneumonia did not seem to perceptibly increase,

while the mortality in pneumonia complicated by empyema was considerably higher, nearly double that of the preceding months.

The mortality tables of the various conditions follow:

	Pneumonia	Primary Empyema	Measles Empyema	Bronchopneumonia and Empyema	Pneumonia and Empyema	Total Empyema
January	16.7	20	64.3	22.2	30.0	44.9
February	11.1	50	20.0	14.3	22.0	27.8
March 1 to 27	14.2	00	00.0	†42.8	42.8	30.0
*March 27 to May 1	14.3	50	50.0	50.0	50.0	51.3
May	12.0	00	100.0	33.3	16.7	36.4
Total Sept. 25 to June 1, 1918	12.9	22.7	61.5	35.6	38.2	40.5

* Several factors at this time, from March 27 to May 1, had important bearing on the character and severity of the condition and on the kind of operative procedure instituted. The greater number of these cases were newly drafted men, some of whom still showed the effects of previous alcoholism, one case having delirium tremens with pneumonia and empyema. The infection was severe and their resistance was definitely low, several patients dying within 3 days after entrance to the hospital. The cases were very septic at the onset, as in January when the same conditions held though to a less degree.

† Of the 3 fatal cases, 2 were treated by costectomy, the third by aspiration.

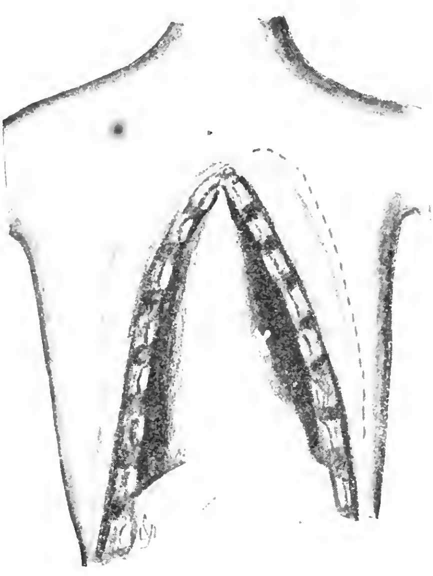
The cases of primary empyema and of empyema following measles are so few that by months the mortality figures are of little value. The totals for the period up to June 1, are based on sufficient number of cases to give a more reliable rate for these conditions.

TABLE 3

ADMISSIONS AND DEATHS ACCORDING TO LENGTH OF SERVICE

Pneumonia (Totals of Columns 6 and 15, Table 2)	1/12 Service			2/12 Service			1/12 and 2/12 Service Combined			All Others		
	Cases	Deaths	% Mortality	Cases	Deaths	% Mortality	Cases	Deaths	% Mortality	Cases	Deaths	% Mortality
September	1	.....	....	....	.....	....	1	.....	....	1	1	100.0
October	3	.....	....	1	.....	....	4	.....	....	1		
November	3	.....	....	1	.....	....	4	.....	....	1		
December	6	.....	....	....	.....	....	6	.....	....	4		
January	15	2	13.3	11	1	9.1	26	3	11.5	8	2	25.0
February	....	.....	....	1	.....	....	1	.....	....	10	2	20.0
March	6	3	50.0	1	.....	....	7	3	42.9	20	3	15.0
April	44	7	15.9	2	1	50.0	46	7	15.2	36	4	11.1
May	62	7	11.3	....	.....	....	62	7	11.3	13	2	15 4
Totals	140	18	12.9	17	2	12.5	157	20	12.7	94	14	14.9
Empyema (Col. 14. Table 2)												
September	....	.....	....	....	.....	....	....	.....	....	....	.....	....
October	....	.....	....	....	.....	....	....	.....	....	....	.....	....
November	....	.....	....	....	.....	....	....	.....	....	....	.....	....
December	3	2	66.7	....	.....	....	3	2	66.7	1	.....	....
January	15	4	26.7	5	4	80.0	20	8	40.0	9	5	55.5
February	....	.....	....	4	1	25.0	4	1	25 0	14	4	28.6
March	4	2	50.0	3	.....	....	7	2	28.6	20	10	50.0
April	12	6	50.0	3	1	33.3	15	7	46.7	12	4	33.3
May	7	1	14.3	3	2	66.7	10	3	30.0	1	1	100.0
Totals	41	15	36.6	18	8	44.4	59	23	39.0	57	24	42.1

Primary empyema (primary so far as the lung condition is concerned) was found most frequently following measles. In these cases the usual early and persistent signs were those of fluid rather than of consolidation. At necropsy an accompanying bronchopneumonia, however, was present in a great majority of cases, it being impossible to state with any degree of assurance whether the empyema or the pneumonia was the older process. The state of consolidation and the degree of organizattion of the exudate in several cases was so evident, however, as to definitely place the empyema as the primary condition. Further evidence of this priority of empyema was obtained



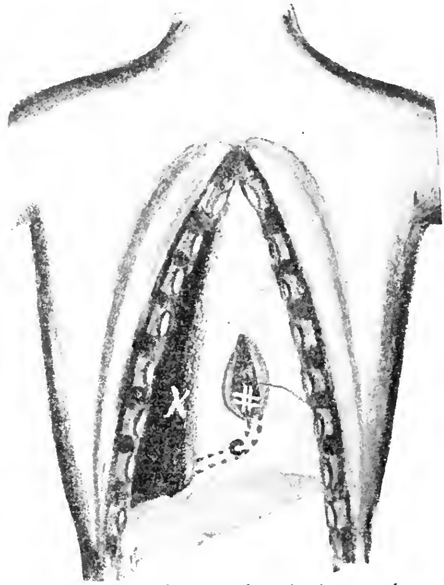


Fig. 1.—Double empyema. Costectomy on the right side and repeated aspiration on the left. X, an anterior pocket which extended from the apex to the diaphragm and contained 500 c.c. of thin turbid fluid; this did not connect with a similar posterior pocket containing 1,400 c.c of a similar fluid. A flat atelectatic lung was attached at the dotted line by fresh adhesions, definitely forming a partition separating the two pockets. Necropsy was done 18 days following the costectomy.

Fig. 2.—Empyema on the right side with pocket formation. Thoracotomy on the right. Both pockets thoroughly drained and cleaned; Dakin's solution had been used. X marks a lateral cavity connecting with Z, the latter being an anterior cavity situated between mediastinal parietal pleura and visceral pleura, anterior to the hilus, by C, canal 2 inches long, the size of the little finger. After operation, a pneumonia developed on the left side. Necropsy was performed 23 days after the operation.

from the distribution of the pneumonia, as the consolidation appearing at the periphery as a shell, apparently secondary to an overlying empyema by an extending infiltration; or even completely encircling an interlobar empyema like a thick orange rind.

The early recognition of empyema depends on constant attention, alertness and accuracy of observation. Repeated critical examinations with careful notations are of great value, especially when graphically indicated on a chest outline allowing a quick and easy comparison of findings. Dulness or flatness with the sense of resistance on percussion, together with the diminution or absence of tactile fremitus, are the most reliable signs. The signs may be looked for in any portion of the chest, as there is no place that the fluid may not accumulate, anterior or posterior, superior or inferior, medial, lateral or interlobar.

The site for thoracentesis is the point where the signs are most definite. Fluid has been aspirated from different pockets in various locations, just inside the right nipple, high and low in the axilla, near the sternum, beneath the clavicle, midscapular region and at the

TABLE 4

TABULATION OF 137 BLOOD CULTURES IN RELATION TO DISEASE AND MORTALITY

	Living		Dead	
	Positive	Negative	Positive	Negative
Pneumonia	2	64	3	11
Measles complicated by pneumonia	0	1	0	1
Measles complicated by pneumonia and empyema	0	0	1	1
Measles complicated by empyema	0	0	1	1
Primary empyema	0	5	0	1
Pneumonia complicated by empyema	3	20	11	11
Totals	5	90	16	26

extreme base. One pocket was located at a depth of 2 inches in the interlobar fissure; another cylindrical pocket extending from the surface to a depth of 15 cm., more of a sinus than a pocket, the size of a finger, with the end of the cylinder at about the angle of the scapula. The pocket formation may be the result of old or new adhesions (Figs. 1 and 2 show the pocket formations).

The roentgen ray has been of very great assistance, not only in determining whether consolidation or pleural fluid was present, but in locating the lesions with precision in a number of cases.

An irregular temperature after pneumonia often led to careful scrutiny of the patient, a collection of pus not infrequently being found interlobar or in some other less usual location. At other times, the temperature being normal for many days, pockets of pus were located by routine careful examination in patients thought to be making an uncomplicated convalescence.

The mortality of cases of empyema as reviewed by months in relation to (1) incidence and mortality of antecedent and accompanying disease (Table 2), and (2) the kind of operative procedure instituted, is instructive.

A negative blood culture does not mean that septicemia is not present. While indeed most of the fatal cases probably had at least a terminal septicemia, it is not believed that these cases at the outset were necessarily predetermined deaths, nor does it seem that it can be fairly stated that because the patients died, they were hopeless from the beginning by reason of there being septicemia present.

From the opening of the hospital, Sept. 27, 1917, to June 26, 1918, there were 116 cases of empyema with 49 deaths. (Two patients died since the tables were completed, one of streptococcus septicemia-positive blood culture, and the other of miliary tuberculosis.)

Of the 67 living, 58 have been discharged from the hospital cured; of the remaining nine, several have required secondary operations but all are doing well and it is expected that they will soon be discharged.

Statistics taken from time to time showing the prevalence of streptococci in the throats of patients entering the hospital and of apparently healthy men throughout the camp are presented. These include data collected from cultures taken for the detection of diphtheria carriers, the routine cultures from patients entering the hospital with suspicious throats, cultures made in a number of patients to determine whether or not they had acquired streptococci during their stay in the hospital, cultures made in 2 empyema and 2 pneumonia wards and a series of cultures made on a company of new draft men.

Table 5 shows the occurrence of streptococci in the throats of patients, and of apparently healthy men on culture to determine the number of diphtheria carriers. The cultures in the latter case were made on Loeffler's serum. After incubation, smears were stained with methylene blue. Incidental to the search for diphtheria bacilli the presence of organisms morphologically streptococci were noted.

A survey of these statistics would indicate that streptococci have been present in the throats of apparently healthy soldiers and patients in the hospital of this camp in a percentage comparable to a percentage which has been found by other workers in healthy individuals in civil life.

Coincident with the arrival of negro troops from Alabama, Type I pneumococcus pneumonia became almost epidemic. During May, 25 cases were diagnosed. Eighteen of these occurred in negro troops.

A large number of these came from 2 barracks (74 and 76). The company occupying Barracks 76 was examined to determine how many men carried Type I pneumococcus. In 98 men, one Type I and eleven Type II organisms were found. The Avery method was used to determine the agglutinable organisms. Blood-agar plates were inoculated from the 24-hour culture. Approximately 15% showed hemolytic colonies of the streptococcus type.

There are also available the results of study of 29 cultures of organisms morphologically streptococci on blood-agar.

TABLE 5

OCCURRENCE OF STREPTOCOCCI IN THROAT CULTURES FOR VARIOUS PURPOSES

	Feb.	Mar.	Apr.
Total number of cultures on diphtheria carriers	853	340	86
Total number containing streptococci	268	205	17
Per cent. containing streptococci	31.4%	60.2%	19.5%

The same technic was used in making cultures on patients entering the hospital. The cultures were made in the receiving office on all patients having suspicious throats:

	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.
Total number of cultures	3	15	107	145	291	135	209
Number containing streptococci	1	13	71	81	203	110	154
Per cent. containing streptococci	33.3%	86.7%	60%	55.9%	69.5%	74.8%	73.7%

Cultures were made in the same way on the patients in 4 measles wards for another purpose, the incidence of streptococci being noted:

	Feb. 5	Feb. 7	Feb. 11	Feb. 13
Total number of cultures	85	34	57	50
Number containing streptococci	54	23	28	30
Per cent. containing streptococci	63.5%	67.6%	49%	60%

A series of cultures were made on a group of patients in the hospital to determine whether or not they acquire hemolytic streptococci in the throat during their stay. For this purpose the cultures were made on plain blood agar plates and the colonies producing wide zones of complete hemolysis and shown to be gram-positive cocci in chains, were diagnosed as hemolytic streptococci:

	Apr. 30	May 2	May 6
Total number of cultures	51	45	35
Number containing streptococci	11	9	2
Per cent. containing streptococci	21%	20%	5.7%

Throat cultures using the latter technic were made on patients in 2 pneumonia and 2 empyema wards:

	Empyema Wards		Pneumonia Wards	
	Ward 14	Ward 16	Ward 21	Ward 22
	Apr. 30	Apr. 30	May 6	May 6
Total number of cultures	21	20	24	34
Number containing streptococci	5	8	8	3
Per cent. containing streptococci	20%	40%	33%	8.5%

A company of new draft men was cultured shortly after arrival and every 7 days thereafter for 3 successive cultures. These cultures were made on Loeffler's serum, smears stained by methylene blue:

	Apr. 29	May 6	May 13	May 20
Total number of cultures	100	91	85	65
Number containing streptococci	39	33	40	27
Per cent. containing streptococci	39%	33%	47%	41%

The results of sputum typing is shown in Table 7. The Avery method with the exception of a few cases has been relied on for the reason that mice were not available and because it is a more simple and economical procedure than the mouse method.

Blood cultures for the most part have been sterile. The technic has been to inoculate from 5-10 c c of blood into 200-300 c c of glucose

TABLE 6

STREPTOCOCCUS CULTURES

Date	Source	Length (Aver.)	Blood-Agar Plate
Feb. 4	Chest fluid	6–12 cocci	Hemolysis complete
Feb. 4	Throat	10–20 cocci	Hemolysis complete
Feb. 5	Sputum	20–30 cocci	Green producing
Feb. 6	Throat	30–60 cocci	No hemolysis
Feb. 6	Throat	20–30 cocci	No hemolysis
Feb. 6	Throat	10–20 cocci	No hemolysis
Feb. 6	Blood culture	8–16 cocci	No hemolysis
Feb. 6	Chest fluid	8–16 cocci	No hemolysis
Feb. 7	Sputum	8–16 cocci	Hemolysis complete
Feb. 7	Sputum	8–16 cocci	Hemolysis complete
Feb. 10	Appendix	8–16 cocci	Hemolysis complete
Feb. 15	Chest fluid	10–25 cocci	No hemolysis
Feb. 15	Throat	8–16 cocci	No hemolysis
Feb. 15	Chest fluid	8–16 cocci	Hemolysis complete
Feb. 15	Sputum	8–16 cocci	No hemolysis
Feb. 15	Sputum	10–30 cocci	Hemolysis complete
Feb. 18	Blood, postmortem	10–30 cocci	Hemolysis complete
Feb. 18	Blood, postmortem	10–30 cocci	Hemolysis complete
Feb. 26	Blood, during life	10–30 cocci	Hemolysis complete
Feb. 26	Chest fluid	10–30 cocci	Hemolysis complete
Feb. 28	Sputum	10–18 cocci	Hemolysis complete
Feb. 28	Sputum	10–18 cocci	No hemolysis
Feb. 28	Axillary abscess	10–18 cocci	Hemolysis complete
Feb. 28	Chest fluid	Moderate	Hemolysis complete
Mar. 18	Chest fluid	Long	No hemolysis
Mar. 20	Sputum	Short	No hemolysis
Mar. 20	Blood culture	Long	Hemolysis complete
Apr. 1	Chest fluid	Long	Hemolysis complete
Apr. 1	Chest fluid	Long	Hemolysis complete

Total number of cultures	29
Number showing complete hemolysis	17 or 58%

TABLE 7

BACTERIOLOGIC STUDY OF SPUTUM

	Type I	Type II	Type III	Type IV	Streptococcus		B. Influenzae	B. Capsulatus	Undetermined	Total
					Alone	With Type IV				
January	0	6	0	21	3	6	0	0	0	36
February	0	2	0	3	15	1	0	0	0	21
March	0	1	0	2	12	0	0	0	1	16
April	1	5	0	0	56	0	0	0	37	99
					Hemolytic	Non-hemolytic				
May	26	11	0	23	11	7	4	8	0	90
Total	27	25	0	49	111		4	8	38	262

broth which is then shaken. The cultures were then incubated and examined at the end of 24, 48 and occasionally 72 hours. Table 8 is a summary of this work:

TABLE 8

BLOOD CULTURES

Number of cases with no culture	148
Number of cases with culture	146
Total number of cases	294
Number of negative cultures	133
Number of positive cultures	21
Per cent. positive	13%

Mortality of cases showing positive blood cultures.

		Cases	Mortality
Streptococcus		17	89%
Pneumococcus	Type I	2	00
	Type II	1	00
	Type III	0	00
	Type IV	1	100%
Total		21	81%

The predominating organism has been a streptococcus. The pneumococcus has been demonstrated but 4 times. The streptococcus present has been a long chain organism, considered in the early cases to be identical with the hemolytic. In the majority of instances when grown on blood agar these organisms have produced wide zones of complete hemolysis about colonies. Since May 1, every streptococcus found in a pleural exudate has been shown to produce complete hemolysis on blood agar. To further identify these organisms more recent cultures have been grown on blood agar and in sugars. Twenty-two cultures from as many cases have been examined in this way. Three of these have been pneumococcus infections. The other 19 all produced wide zones of complete hemolysis, and failed to ferment mannite and inulin. In these respects they may be considered identical organisms.

Leukocyte counts have been made in the majority of cases. The average for the total 142 counts was 17,000. The lowest count was 3,000, the highest 58,000. In a large number of cases the leukocytosis has been greater than 30,000 especially at the time when the empyema was discovered. No constant relationship existed between the degree of the leukocytosis and the mortality. Both the patients having the counts of 3,000 and 58,000 died.

Seventy-one blood cultures were made on 55 cases of empyema, 18 were found to contain streptococci, 3 pneumococci, positive 28.5%. Six of the cultures yielding streptococci were obtained postmortem.

Of the remaining, 6 patients having a streptococcemia antemortem lived, 6 died; one having pneumococcemia; one died. One pneumococcus was demonstrated postmortem.

The sputum was typed in 47 cases of pneumonia complicated by empyema. In 33, streptococci were demonstrated; in 13, a pneumococcus; 4 had Type II organisms, the others Type IV. In one case a diagnosis was not arrived at.

The organisms found in the chest fluid have been in 91 cases the streptococcus; in 2, pneumococcus Type II; in 2, pneumococcus

TABLE 9

CULTURES GROWN ON BLOOD AGAR AND SUGARS

No.	Source	Blood Agar	Dextrose	Lactose	Mannite	Saccharose	Inulin
1	Chest fluid	Hemolysis	+	+	—	+	—
2	Chest fluid	Hemolysis	+	+	+	+	—
3	Chest fluid	Hemolysis	+	+	—	+	—
4	Chest fluid	Hemolysis	+	+	—	+	—
5	Chest fluid	Hemolysis	+	+	+	+	—
6	Chest fluid	Hemolysis	+	+	+	+	—
7	Chest fluid	Hemolysis	+	+	+	+	—
8	Chest fluid	Hemolysis	+	+	+	+	—
9	Chest fluid	Hemolysis	+	+	—	+	—
10	Chest fluid	Hemolysis	+	+	+	+	—
11	Chest fluid	Hemolysis	+	+	—	+	—
12	Chest fluid	Hemolysis	+	+	+	+	—
13	Chest fluid	Hemolysis	+	+	—	+	—
14	Chest fluid	Hemolysis	+	+	—	+	—
15	Chest fluid	Hemolysis	+	+	—	+	—
16	Chest fluid	Hemolysis	+	+	+	+	—
17	Chest fluid	Hemolysis	+	+	—	+	—
18	Chest fluid	Hemolysis	+	+	—	+	—
19	Chest fluid	0	+	+	+	+	+
20	Chest fluid	0	+	+	+	+	+
21	Chest fluid	0	+	+	+	+	+
22	Chest fluid	0	+	+	+	+	+

– = acid production; — = no reaction.

Four of these cultures were considered pneumococcus because of the morphology, fermentation of inulin and production of methemoglobin.

Number 19 agglutinated with Type 2 antipneumococcus serum.

Number 21 agglutinated with Type 1 antipneumococcus serum.

Type IV. In 9 cases no organisms were demonstrated in the pleural exudates.

In general, the findings in sputum, blood culture and chest fluid have corresponded. In only 3 cases have the organisms in the blood differed from those found in the chest fluid. Two of these had Type II pneumococci in their blood with streptococci in their chest fluid. In one a pneumococcus Type IV was found in the chest fluid with a streptococcus in the blood stream.

There have been 37 necropsies, in a series of 71, on cases of pneumonia and empyema, empyema occurring in 29.

The results show that the infection in these cases has not been limited to pleural cavities, but has in the majority of instances, been found in various locations. These facts together with the results of the blood cultures would seem to indicate that, at least in fatal cases, the infection was systemic. A streptococcus has been identified in the majority. Measles was a factor in 12 cases. In these 12 cases, empyema followed or was associated with bronchopneumonia in all except two.

The most constant picture at necropsy has been a serofibrinopurulent pleuritis associated with bronchitis and bronchopneumonia. There have been 15 cases with right sided lesions, 10 with left, 4 bilateral.

In the early cases the pleural lesion consists of a large amount of exudate which compresses the lung against the hilus to a varying degree. The pleural surfaces have been covered uniformly with a layer of fibrin the thickness of which depends on the age of the process. The interlobar fissures have been found sealed tightly. In a few cases a pocket of pus has formed in the fissure, the exudate between the lobes becoming purulent. In other cases the base of the lung has been bound firmly to the diaphragm, a dome-shaped pocket of pus forming in this location. Pocket formation has been shown far more frequently clinically than at necropsy. In old cases the pleural cavities become obliterated and the undrained pus enclosed by thick walls of organizing exudate. The cause of death in case of long duration has usually been due to an extending process. One patient died with a marked acute glomerulo-nephritis. The rapidly developing fulminant cases rarely have any changes other than those found in the chest to which the cause of death may be attributed.

The lung lesions in these cases have been almost identical with the descriptions of MacCallum. With large amounts of fluid or pneumothorax in the operated cases and corresponding atelectasis, usually the lung of the opposite side has presented the most typical picture of the pneumonic process. When the fluid has not been too great, a lobe on the same side may present a typical picture. Bronchopneumonia has been associated with empyema in 22 cases and in 4 others no pneumonic process was described. The lobar pneumonia encountered has not been unusual.

The bronchopneumonia encountered presents several striking features. In general, it has been extensive and patchy in distribution, involving a whole lobe or lung so that it is easy to see how such a case clinically would be considered a lobar process. The lung has had a

fairly firm consistency, however, the type of consolidation has not been that of lobar pneumonia. On section innumerable, widely scattered, deep red, dry, elevated areas appear throughout the lobe or lung between which apparently normal alveolar tissue could be demonstrated. In such a section small abscesses would be encountered, but no direct connection between these abscesses and the bronchi could be shown. In later stages the lung tissue was distinctly yellowish or grayish representing what appeared to be a resolving process. The congestion and edema in some cases was so marked as to give the lung the appearance of raw meat. In the few cases examined microscopically the pneumonic process is described as peribronchial in type.

Peritonitis occurred in 6 cases. In 2 the appendix, according to history and clinical course, was the primary lesion, the peritonitis developing subsequently. In one of these the pleural lesion was recognized antemortem; in the other postmortem. In 2 cases no focus was found within the abdomen. In these the widespread infection was attributed to the virulence of the organism. In 2 cases a localized diaphragmatic peritonitis was demonstrated, particularly over the liver, the pleural lesions in both instances being on the right side. Though no conclusive evidence was found it seems probable that in these cases the peritoneal infection took place by direct extension through the diaphragm.

Pericarditis occurred 7 times. In one case it was extremely slight, manifested by a slightly turbid fluid and a few threads of fibrin on the auricles. The others have been described as hemorrhagic, serofibrinous or purulent. They are undoubtedly only a manifestation of a general septicemia and as such should be regarded as terminal lesions.

Mediastinitis, manifested by purulent exudate in the mediastinal tissues, has been encountered in 3 cases.

SUMMARY

Respiratory infections in 1917 and 1918 occurred among troops in waves, the greatest severity of the diseases being at the height of each wave. Not only were pneumonia and empyema more frequent and severe, but also tonsillitis, pharyngitis and bronchitis.

The most serious infections were characterized by septicemia with empyema, peritonitis, phlegmon and rarely arthritis; and by pulmonary infection (pneumonia, lobular or lobar in distribution) with consecutive empyema.

While patients who were passing through measles infection were more severely affected by the streptococcal invasion, the 2 infections should be regarded as separate even though they were coincident. The empyema and pneumonia were not the result of the measles infection but of the prevailing severe respiratory infections of that period. The marked freedom from complications of the group of measles treated during the fall and winter of 1917 when general streptococcal infections were absent in camp, and the severe wave of pneumonia and empyema (streptococcal) of April, 1918, when measles was on the wane and which involved for the most part soldiers who had not had measles, are points in support of this view.

In a group of 115 consecutive cases of pneumonia and empyema a definite history of an acute antecedent bronchitis, pharyngitis or tonsillitis was obtained in over 80% of the cases.

In the majority of cases of empyema, a streptococcus has been the causative organism. When grown on blood agar it has been shown to be usually hemolytic. During the period of prevalence of empyema, streptococci were demonstrable in the majority of the throats of healthy soldiers (including fresh recruits) as well as of patients entering the hospital with respiratory infections.

The clinical manifestations of empyema include rapid and profound toxemia, the quick formation in many cases of large amounts of pleural exudate, and a marked tendency to pocket formation by old and new adhesions in atypical locations.

The mortality was highest (61.5%) in the cases of empyema following measles, and the lowest (22.7%) in the cases in which empyema was the primary condition. (Cases following measles are not designated primary.) In empyema complicating pneumonia the mortality was 38.2%; the mortality of all cases of empyema was 40.5%.

At necropsy the outstanding features have been the finding of widespread lesions with a tendency to involve serous membranes, the occurrence of a severe bronchitis in the majority of cases, and a type of bronchopneumonia so distributed as often to closely resemble a lobar process.

Early recognition of empyema is important, especially in the acute fulminant types of cases. The diagnosis depends on close attention to clinical progress, frequent observations accurately made and recorded, and proper interpretation of physical signs. Dulness, the sense of resistance on percussion, and the diminution or absence of tactile fremitus are the most reliable signs.

The relative incidence of empyema complicating pneumonia was high, one month 50% of pneumonia cases developing empyema.

Of 830 cases of measles (including rubella), 4.4% developed pneumonia or empyema; of this group, empyema was the primary condition clinically in 70% of the cases.

The control of such epidemics would appear to depend on the sanitation in barracks as regards ventilation; adequate space and proper separation, especially of those even slightly affected with respiratory disease; on line officers not allowing overheated men to stand inactive in cold winds; on not indiscriminately giving triple typhoid inoculations at the very entrance of the soldier to camp without regard to any respiratory infection he might have at the time; on careful attention to overexertion and exposure of soldiers having mild respiratory disease, and on close attention to the details of equipment to ensure adequate clothing for the unacclimated raw recruit on his arrival.

THE VIRULENCE OF THE TUBERCLE BACILLI ISOLATED FROM THE SPUTUM

H. J. Corper

From the Laboratories of the Municipal Tuberculosis Sanitarium, Chicago

It is rather singular that the tubercle bacillus was one of the first pathogenic micro-organisms isolated in pure culture, and yet its virulence in the sputum, the most important route of exit from the body in man, has been studied only in an unsatisfactory manner considered from a hygienic and sanitary standpoint.

Villemin as early as 1865 noted that tuberculous material from man and cattle was not equally infectious to certain animals, but even Koch paid no heed to this important fact until it was indelibly impressed on the scientific world by Theobald Smith in 1898 when he presented his proof absolutely differentiating the human and bovine tubercle bacillus. On account of the lack of this data virulence tests, such as they may have been before this time, were of no practical value.

Smith[1] studied the tubercle bacilli obtained from the sputum of 7 consumptives, the material from a dog infected by his master, 6 bovine specimens, 1 from a swine, 1 from a cat and 1 from a horse by passage through guinea-pigs and subsequent cultivation on dog's serum. His emulsions for inoculation were made rather crudely, "equal in density to that of a bouillon culture of typhoid bacilli 20-24 hours old and nearly as homogeneous." He noted a sharp distinction between bovine and human bacilli when injected intravenously into rabbits, the former causing early death (17-21 days) and generalized tuberculosis, while the latter was so innocuous that the rabbits lived over 35 days with only a pulmonary tuberculosis in most cases. Among the sputum forms he noted variations in size, in capacity for cultivation and in pathogenic activity. In a paper read before the American Climatological Association in 1898,[2] he points out 2 important factors active in the production of disease; the virulence or pathogenic activity of the organism, and the resistance of the infected individual, and in criticizing Arloing's conclusions regarding the difference between bacilli

Received for publication June 5, 1918.

[1] Jour. Exper. Med., 1898, 3, p. 451.

[2] Smith, Theobald: Tr. Am. Climatolog. Assn., 1898, 14, p. 193.

harbored in pulmonary and surgical forms of tuberculosis, deduced by the injection of infected tissues, points out the fact that the number of bacilli introduced are of utmost importance, yet his own work only crudely took amounts into consideration.

Vagedes,[3] who examined the bacilli from 30 cultures (6 of these from human sputum) by injecting them into rabbits fell into the same pitfall, as did also Lartigau,[4] who believed he saw a definite relation between the virulence of the bacilli and the clinical course of the disease; chronic course associated with a low virulent bacillus and rapid course and miliary tuberculosis associated with high virulence of the bacilli. Veszpremi[5] examined 10 cultures (2 of them laboratory strains and 8 recently isolated from cases of pulmonary tuberculosis) by intravenous injection into rabbits, using 4 rabbits for each culture, two receiving about ½ mg. and the other two 3 mg. He concluded as a result of his observations that tubercle bacilli freshly isolated from cases of pulmonary tuberculosis reveal definite differences in virulence. Vagedes[6] in a brief polemic criticizes Veszpremi's work because the latter obtained his cultures by passage through guinea-pigs before culture, and comments on the fact that their conclusions are alike with regard to the difference in virulence. Moeller[7] studied a small number of cultures obtained from various types of tuberculosis and even tho he believed he saw a difference in the virulence of these bacilli he saw no relation between the type of disease and the virulence for guinea-pigs. Dorset[8] studied 12 cultures obtained by passage through guinea-pigs, 9 of these from human sources, of which 2 were from sputum and 3 from lungs direct. A rabbit and a guinea-pig were inoculated from each culture on egg medium and conclusions were drawn on the basis of the duration of life and macroscopic and microscopic findings after death. A suspension equal in density to a 24-hour typhoid culture was injected in 0.5 c c amounts. He concludes that there is a considerable variation in the virulence of different strains of human tubercle bacilli for rabbits and guinea-pigs. Fraenkel and Baumann[9] obtained 37 cultures from human sources, 30 of these from the sputum were isolated with Heyden agar directly. Their emulsions

[3] Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 276.
[4] Jour. Med. Research, 1901, 6, p. 156.
[5] Centralbl Bakt., I, O, 1903, 33, p. 176 and p. 255.
[6] Ibid., p. 679.
[7] Ztschr. f. Tuberk. u. Heilstätenw., 1903-4, 5, p. 5.
[8] U. S. Dept. Agricult. 21st Ann. Report Bur. An. Ind., 1904, p. 159.
[9] Ztschr. f. Hyg. u. Infektionskr., 1906, 54, p. 246, and 55, p. 327.

were made from 4-week old cultures which had been continued on coagulated glycerol cow serum. Their experience with rabbits, rats and mice led them to conclude that they were unsuitable for virulence tests, especially for revealing differences in pathogenic properties of the tubercle bacilli. Guinea-pigs were found, however, to be satisfactory. They claim that of some cultures even one bacillus would cause tuberculosis, but the proof of this was lacking. Their reasoning was by analogy with B. prodigiosus, it having been found that there is one bacillus in one 10,000 millionth to one 100,000 millionth of a mg. (their paper is not entirely clear on the amount referred to or used in their experiments). They noted no appreciable decrease of virulence of cultures grown on artificial mediums for 6 years and conclude that the range of virulence of their cultures lay between one 1,000 millionth and one 100,000 millionth of a mg. Marmorek,[10] in 1906, called attention to the fact that as a criterion of the infectious power of tubercle bacilli, as a measure of their virulence, it is necessary to note the distribution of the anatomic lesions and only in a very insignificant manner consider the duration of life. If a series of test animals are inoculated in the same way and at the same time there is found after a definite time period, an equal amount of distribution of lesions in each animal. The difference in the anatomic lesions is the best gauge of the rapidity of the infection. In a brief summary contribution, Rodet and Delanoe[11] report on 26 cultures obtained from sputum by passage through guinea-pigs and rabbits. They do not give the method of inoculation of the cultures, but conclude that bacilli which were most virulent for rabbits were also most virulent for the guinea-pigs. They believe that they saw a relation between the virulence of the bacilli and the rapidity of the case, low virulence bacilli from chronic prolonged cases of tuberculosis. In a brief presentation, Meyer and Lewis[12] state that the cultural characteristics and virulence for small animals of 4 cultures of tubercle bacilli, 2 from extremely chronic cases and 2 from very acute cases presented no points of distinction. Gilbert and Gregg[13] inoculated individual tubercle bacilli counted by the Barber method and found as a result of the examination of 13 freshly isolated cultures (mostly from sputum) less than 1 month old, that the minimum lethal number

[10] Berl. klin. Wchnschr., 1906, 43, p. 328.
[11] Comp. rend. Acad. d. sc., 1908, 147, p. 500.
[12] Tr. Natl. Assn. Study and Prevention of Tuberculosis, 1913, 9, p. 332.
[13] Med. Rec., 1915, 88, p. 208.

was approximately from 10 to 120 bacilli (less than 10 bacilli were not tested and the time interval allowed for the development of the infection in the guinea-pigs which were used as test animals was also not noted). The authors state that "In spite of the small numbers of cultures as yet studied it is certainly safe to assert that great differences in virulence occur in virulent cultures obtained from various cases. Whether cultures obtained from varied organs vary regularly in virulence remains to be determined."

Webb and Gilbert[14] during the course of experiments on immunity in tuberculosis in which they gave injections of definite numbers of tubercle bacilli found that less than 10 virulent human tubercle bacilli could infect a child. They also injected 60 bacilli of a culture of which 35 bacilli would infect a guinea-pig, into 2 young guinea-pigs, 1 pregnant female, a nursing female and a normal male. The young pigs died in 2½ and 3 months after infection, while the remainder were killed after 5 months, the pregnant female revealing extensive tuberculosis, the nursing female no tuberculosis, and the male a moderate amount of tuberculosis. The authors conclude from these experiments that very young pigs are much more susceptible to tuberculosis than older pigs given definite small numbers of bacilli.

That death in tuberculosis is to a great extent attributable to accident is again well emphasized in a recent article by Krause.[15]

The incidence of infection with bovine tubercle bacilli in pulmonary tuberculosis as determined from the sputum is summarized up to 1916 in a contribution by Wang.[16] Of a total of 998 cases, "the bacilli from 3 cultures were found to be of a mixed type, both 'human' and 'bovine,' and from 4 of a pure 'bovine' type."

Since there are a number of unsettled points which are of definite import in determining the virulence of tubercle bacilli, preliminary experiments were carried out to either prove these as unessential precautions or if possible find and institute the necessary ones.

In order to determine whether there is an appreciable difference in the reaction of guinea-pigs to tubercle bacilli at various ages the following experiment was performed:

Eight male guinea-pigs from the same stock were selected for use; 4 about 3 months old and 4 about 1 year old. They were all injected subcutaneously in the left lower quadrant of the abdomen with 0.3 c c of a good emulsion of a culture V of virulent human tubercle bacilli containing 0.000,001 mg. of

[14] Jour. Am. Med. Assn., 1914, 63, p. 1098.
[15] Am. Rev. of Tuberculosis, 1917, I, p. 65.
[16] Lancet, 1916, 2, p. 417.

TABLE 1

THE ANATOMIC DISTRIBUTION OF TUBERCULOSIS IN MALE GUINEA-PIGS OF DIFFERENT AGES

Days Sectioned	Young Pigs	Mature Pigs
42	++*	++
	+++	++
62	+++	++++
	+++	++++

* Throughout this paper — designates no microscopic anatomical tuberculosis; + distinctly enlarged local and slightly enlarged retroperitoneal glands; ++ enlarged local and retroperitoneal glands and slight involvement of the spleen; +++ enlarged local and retroperitoneal glands, spleen markedly involved, the peritracheal glands enlarged and the lungs slightly involved; ++++ massive involvement of all the glands, the spleen, lungs and liver.

culture per c c.* They were then kept under identical conditions of feeding, etc., during the entire experimental period. Two of each set were killed 42 days after infection and the other two after 62 days.

It is to be noted from the foregoing experiment that when small numbers of human tubercle bacilli are injected subcutaneously into male guinea-pigs of 3 months and 1 year old there is no marked difference in the amount of anatomic involvement obtained on section after 42 and 62 days.

TABLE 2

VIRULENCE OF HUMAN TUBERCLE BACILLI FROM CULTURES AT VARIOUS TIMES

Age of Cultures in Months	Injections in Mg.*	Cultures 1	Cultures 2	Cultures 3
1	0.1	+++†	++++	++++
	0.001	++++	+++	++++
	0.000,001	+++	++	+++
	0.000,000.01	+	—	+++
2	0.001	+++	+++	++++
	0.000,001	+++	+++	+++
	0.000,000,01	—	—	+++
3	0.001	++++	+++	++++
	0.000,001	+++	++	++
	0.000,000,01	+	—	++

* All the guinea-pigs were sectioned about 2 months after infection. One guinea-pig was used for each dilution.
† See previous table for value of these markings.

In order to determine whether there is an appreciable difference between the virulence of human tubercle bacilli, which had been isolated by Petroff's method,[17] when kept in the incubator at 37-38 C. with due precautions against drying out, etc., at 1 and 3 months (satisfactory growths for virulence tests are in most cases only obtained after 1-2 months' incubation subsequent to inoculation of the sputum) the following experiment was performed:

* Organism V only produced tuberculosis of the local glands in amount of 0.000,000,01 mg. after 2 months.

[17] Jour. Exper. Med., 1915. 21, p. 38.

Two cultures of human tubercle bacilli were injected subcutaneously into male guinea-pigs all about the same size and about 6 months old after the cultures were 1, 2 and 3 months old, in amounts as specified in Table 2.

That the virulence of the bacilli is not appreciably altered on a second seeding into a gentian violet egg medium tube (Petroff) is shown in Table 3.

TABLE 3

Virulence of Human Tubercle Bacilli on Primary and Second Culture on Petroff's Medium *

		Cultures	
—		1 † (2356)	2 (204)
Primary	0.1	++++‡	++++
	0.001	++++	+++
	0.000,001	+++	+++
	0.000,000,01	++	++
Second	0.000,001	+++	+++
	0.000,000,01	+	++
	0.000,000,000,1	—	++

* Culture 1 was transferred to the second Petroff tube six months and Culture 2 four months after the primary culture was started.
† All the guinea-pigs were sectioned about 2 months after infection. One male guinea-pig about 6 months old was used for each dilution.
‡ See Table 1 for value of these markings.

A glance at Tables 2 and 3 reveals that virulent human tubercle bacilli on Petroff's medium do not appreciably alter their virulence for guinea-pigs whether tested after 1 or 3 months, provided no detrimental influences are introduced such as drying out, light, etc., and that a second culture on Petroff's medium produces bacilli of equal virulence for guinea-pigs to that of the primary.

In preparation of the cultures directly from the sputum by Petroff's method they are exposed to the action of an equal volume of 3% NaOH for from 20-30 minutes at 37 C. Soparker[18] found that tubercle bacilli would still grow well after exposure to 2½% NaOH (an equal volume of 5%) for 1-2 hours at 37 C.; 3 or more hours' exposure caused a definite reduction in colonies while some even resisted destruction for as long as 24 hours. He did not, however, determine the effect of such treatment on the virulence of the bacilli, and for this purpose the following experiments were performed:

Three freshly isolated cultures of human tubercle bacilli were carefully emulsified and to 0.1 c c of various amounts, after reserving equal quantities in salt solution for control injections, were added 1 c c of 3% NaOH solution. These mixtures were all kept at 37 C. for 1 hour when they were neutralized,

[18] Indian Jour. Med. Research, 1916, 4, p. 28.

carefully avoiding overheating, with hydrochloric acid using phenolphthalein as indicator, and then were injected into male guinea-pigs about 6 months old.

Two freshly isolated cultures of human tubercle bacilli were likewise used but treated with 6% NaOH for 1 hour.

The results of these 2 experiments are recorded in Table 4.

TABLE 4

THE EFFECT OF EXPOSURE TO 3 AND 6% NAOH FOR 1 HOUR ON THE VIRULENCE OF HUMAN TUBERCLE BACILLI

Amount of Tubercle Bacilli Injected, in Mg.	Cultures									
	1		2		3		4		5	
	Control	3% NaOH	Control	3% NaOH	Control	3% NaOH	Control	6% NaOH	Control	6% NaOH
0.1	+++*	++	++++	++++	+++	+++	++†	+	+++	+++
0.001	++	+++	+++	++++	++	+++	++	—	+++	++
0.000,001	++	+	++	++	++	++	+++	—	+++	++
0.000,000,01	—	—	++	++	++	++	++	—	++	++

* The guinea-pigs were sectioned about 2 months after inoculation.
† It is noted in some cases that the larger doses of tubercle bacilli not infrequently result in less anatomic involvement than the smaller doses; this may be due to the fact that in the larger doses ulcers are frequently formed locally resulting in discharge of a considerable part of the tuberculous mass.

Since in the Petroff method only an equal volume of 3% NaOH solution is added to the sputum and incubation is only continued for 30 minutes, it is safe to conclude that this cannot materially affect the virulence of the bacilli in the original sputum. NaOH solution, 3%, at 37 C. for 1 hour has no appreciable effect on the virulence of human tubercle bacilli for guinea-pigs, while 6% may destroy the virulence of some cultures within 1 hour.

In order to determine whether there might be any difference in the virulence of the cultures of human tubercle bacilli isolated from the sputum of the same patient at different times by the method employed or in different cultures from the same sputum, the sputa from 4 cases were thus studied with the results given in Table 5.

From these experiments it is to be noted that the virulence of human tubercle bacilli isolated from the same sputum but grown on 2 different culture tubes do not differ appreciably within the limits of error of this method as tested in guinea-pigs. Likewise the bacilli isolated from the sputum of the same patient at different intervals (1 and 3 months) give concordant results (within the limit of error of the method) in virulence tests on guinea-pigs.

A series of preliminary experiments were performed in an attempt to note in a general way just how common a virulent or low virulent

TABLE 5

EXAMINATION OF DIFFERENT CULTURES ISOLATED FROM THE SAME SPUTUM AND FROM THE SAME PATIENT AT DIFFERENT TIMES

Sputum		Results of Guinea-Pig Inoculations, Mg.			
		0.1	0.001	0.000,001	0.000,000,01
1	Initial 3 months later	+++ ++++	++ +++	++ +++	+ ++
2	Initial 1 month later	++++ +++	+++ +++	+ ++	— —
3	Both taken at same time	+++ ++++	lost +++	+++ +++	+ +
4	Both taken at same time	++++ ++++	++++ ++++	+++ +++	++ +++

tubercle bacilli could be isolated by culture from the sputum of consumptives positive by staining methods. Ten freshly isolated cultures were tested in dilutions as low as 0.001 mg. by subcutaneous injection, into guinea-pigs. Since not a single low virulent bacillus was found in this series it was decided to perform a large series of tests and carry the dilutions to a point at which only a small number of bacilli were present in the highest dilution. For this purpose the following technic was pursued throughout the remainder of the virulence tests:

The freshly isolated culture of tubercle bacilli on Petroff's medium (egg medium plus 1 : 10.000 Grübler's gentian violet from 1-2 months old) was weighed on a delicate balance weighing accurately to 0.1 mg. in a graduated 15 c c centrifuge tube and carefully emulsified, using a roughened glass rod for this purpose, first without and later gradually adding salt solution until there resulted an emulsion without any lumps (this requires about 20-30 minutes of careful grinding). This emulsion was then diluted so that 1 c c contained 1 mg., stirred well and allowed to stand 5 minutes when 1 c c drawn from the center of the emulsion was put into a second graduated centrifuge tube and diluted to 10 c c with sterile salt solution. Thus were made the following dilutions: 0.1, 0.001, 0.000,001, and 0.000,000,01 mg. contained in a c c. All the foregoing amounts were injected into male guinea-pigs about 4-7 months old, as soon as preparation was completed, and 0.1 mg. was injected intravenously into 1 or 2 mature rabbits to determine the type of bacillus, human or bovine. In order to get an approximation of the number of tubercle bacilli present in the above mentioned amounts, 10 of the 0.1 mg. dilutions were mixed as soon as prepared (after standing the tubercle bacilli would settle out and again become lumped so that simple mixing did not suffice in producing a good uniform emulsion) with an equal volume of oxalated blood of known red corpuscle content. This mixture was spread, as in making blood smears, on about a dozen slides, allowed to dry and fixed by methyl alcohol. The bacilli were then stained by the ordinary carbolfuchsin staining method either cold or warm. Usually a few good slides were obtained from each set and the proportion of tubercle bacilli to red cells noted. Various bloods were used

for this purpose but none possessed advantages over the others. The tubercle bacilli were usually found in small clumps of from 2-10 bacilli if the emulsion was well prepared.

The 10 emulsions counted revealed figures between 5.01 billion and 7.56 billion tubercle bacilli per mg. original culture. Thus the lowest dilution injected into the guinea-pigs contained approximately 50-75 tubercle bacilli. A total of 90 cultures from sputum were thus tested for their virulence within 1-2 (or in a few cases 3) months after isolation on Petroff's medium. The patients from whom the sputum was obtained for study of virulence ranged in age from 13-59 years, and were classified according to the National Association Classification into incipient, moderately advanced, and far advanced cases. As nearly as this could be approximated the duration of the disease and the rapidity of its course was considered.

TABLE 6

CLASSIFICATION OF CASES AND RESULTS OF ANIMAL INOCULATION OF CULTURES *

Classification†	Rabbit (Intravenously)					Guinea-Pig (Subcutaneously)											
	0.1 Mg.					0.000,001 Mg.							0.000,000,01 Mg.			
						Not Examined below this Amount			Examined below this Amount							
	—‡	+	++	+++	++++	+	++	+++	+	++	+++	++++	+	++	+++	++++
Incipient																
Rapid course		1	1				1							1		
Slow course	1	3		2					1					4	1	
Moderately advanced																
Rapid course	1	1		1						1			2			
Slow course	3	7	5	1	1		1			1		1	6	6	2	
Far advanced																
Rapid course	9	10	1	5	2	1	1		1	1	1		14	7	1	
Slow course	4	10	9	3	7		2	4	1	2			8	5	7	2
Totals (not including avirulent and bovine organisms).....	86					10			10				66			

* In a total of 90 cultures examined the following deviations from those tabulated were found:

One virulent organism which produced only local tuberculosis even in 1 mg. amount in the guinea-pig (none in the rabbit) obtained from a moderately advanced case running a very slow course.

One moderately virulent producing a + tuberculosis in the guinea-pig in 0.001 mg. amount (none in the rabbit) obtained from a far advanced case running a very rapid course.

One bovine organism from a far advanced case of very slow course which produced a + tuberculosis in the guinea-pig in 0.000,000,01 mg. amount.

One organism from a far advanced case with rapid course which produced a + tuberculosis in the guinea-pig in 0.000,000,01 mg. amount, and in 0.1 mg. amount intravenously in the rabbit produced after 2 months marked tuberculosis of the lungs, kidney and omentum, but not death.

† This classification is only an approximation as nearly as could be attained from the available clinical data on the cases.

‡ These markings indicate the amount of pulmonary involvement (the main, and generally only, organ affected macroscopically in the rabbits within 2 months after intravenous injection of 0.1 mg. human tubercle bacilli).

Reference to Table 6 and the appended notes reveals that of 90 cultures of tubercle bacilli isolated directly from the sputum of consumptives, 88 were of the human variety and, of these, 86 proved to

be virulent for guinea-pigs in 0.000,001 mg. amounts; 10 cultures were not examined below this amount and, of the remaining 76 that were examined in 0.000,000,01 mg. amounts, 66 produced definite metastatic tuberculosis within 2 months after inoculation. It is interesting to note that of the 2 low virulent organisms isolated from the sputum, 1 was absolutely innocuous to guinea-pigs even in 1 mg. amounts and was obtained from a moderately advanced case of very slow course which became a closed case under observation, and never presented appreciable constitutional symptoms. On the other hand, the other organism of low virulence which produced only a slight tuberculosis in guinea-pigs in 0.001 mg. amounts within 2 months was obtained from a far advanced case of very rapid course having passed from incipient to far advanced within 4 months and presenting severe constitutional symptoms until the performance of artificial pneumothorax. It is also to be noted that there does not seem to be any relation between the virulence of the tubercle bacilli for guinea-pigs or rabbits and the severity or rapidity of the disease in man. There seems to be just as little relation between the infection in rabbits and in guinea-pigs.

SUMMARY

The tubercle bacilli isolated by Petroff's method from the sputum of 90 cases of pulmonary tuberculosis in man, of which 8 were incipient, 20 moderately advanced and 62 far advanced, were found to be of the human variety in 88 of these cases. Of these 88 cultures examined for virulence to guinea-pig (by subcutaneous injection), 86 proved to be virulent in amounts of 0.000,001 mg., 10 of these were not examined below this amount and 66 produced tuberculosis (beyond the local glands) in guinea-pigs within 2 months in 0.000,000,01 mg. amounts. One of the 2 low virulent tubercle bacilli only produced a slight tuberculosis in guinea-pigs in 0.001 mg. amounts within 2 months, while the other produced no tuberculosis beyond the local involvement even in 1 mg. amounts.

No relation was observed between the virulence of the human tubercle bacilli for the guinea-pigs and rabbits, and the rapidity of the disease in man.

No appreciable difference, within the limits of error of the experiments, between the virulence of human tubercle bacilli tested by subcutaneous injection for young (4 months) and mature (1 year) male guinea-pigs was noted on section 42 and 62 days after infection.

Human tubercle bacilli isolated on Petroff's medium do not appreciably alter their virulence for a period of 1-3 months as tested in guinea-pigs provided no detrimental influences are introduced. A second seeding on Petroff's medium within this time produces bacilli of equal virulence to guinea-pigs to the primary.

Treatment of human tubercle bacilli with 3% NaOH solution for 1 hour at 37 C. does not appreciably affect the virulence of the bacilli for guinea-pigs while 6% NaOH for 1 hour at 37 C. will destroy the virulence of some cultures.

The test for virulence of human tubercle bacilli isolated from the sputum of the same patient at different intervals (1 and 3 months) give concordant results in guinea-pigs.

SPECIFIC FATS AS FACTORS IN IMMUNE PROCESSES

C. C. Warden

*From the Hygienic Laboratory of the University of Michigan, Ann Arbor, Mich.**

The work presented in this paper is a continuation of what appeared in a previous article,[1] in which it was shown that the value of antigens in serum tests for the presence of antibody depends on fatty complexes of definite or specific configuration which represent the fatty content of the micro-organisms giving rise to the antibody. The antigens dealt with were the specific fat antigens of the red blood cells of the sheep, the gonococcus, the treponema pallida, the typhoid bacillus, and the cholera vibrio. The methods outlined in the article referred to apply to the present work and need not be repeated, but any radical departure from them will be noted and new methods given.

THE ANTIGEN OF RED BLOOD CELLS OF THE SHEEP

This antigen, consisting of the fats peculiar to these cells in the form of sodium salts, was shown to have the specific power to fix complement in the presence of its appropriate antibody. Granted that the specificity of such a reaction lies in the immune body and the antigen it should be possible by some means to produce the antibody in experimental animals by inoculating them with the antigen. Accordingly this was tried, with success.

The method of preparing the material was first given careful consideration, and then the state or condition of the material and the manner of introducing it. The idea was to imitate as closely as possible the processes of an infection and to bring the antigen in contact with the body fluids of the animal in a state at least bordering on that in which it probably exists in the red blood cells. The first effect desired was that of surface action of the antigen on the plasma of the animal comparable to the effect of the surfaces of the corpuscles themselves, the assumption being that in all cells those substances which are most active in regulating surface tension and surface permeability are found at the surface and consist largely of fat and lipoidal material. The second effect to attain was the education,

Received for Publication June 8, 1918.

* With the collaboration in parts of J. B. Kelly, the Board of Health, Detroit.

[1] Warden, C. C.: Jour. Infect. Dis., 1918, 22, p. 133.

if one may use the word, of the plasma to those surface substances, that is, the antigen, by frequent inoculation in small quantities. The third end was to preserve proper proportions between the ingredients of the antigen and between the antigen and the plasma of the animal.

We have observed that in the fixation of complement by the fat antigens at room temperature, 37 C. and in the refrigerator, cholesterol is probably essential to accurate and clear-cut reactions, and that the best results are obtained when the relative weights of cholesterol and the fat antigen complex approximate 4:1. This ratio applies equally to the syphilitic antigen. In addition, cholesterol and its esters are constituents of nearly all cells. Accordingly, the materials first selected for antigenic purposes were:

1. An aqueous solution of the protein free salts of the specific fats of red blood cells.

2. An aqueous colloidal suspension of protein free cholesterol.

3. An aqueous suspension of typhoid bacilli which had been killed, fixed by 1% formol, washed, and kept in distilled water for a year. This suspension gave no protein reaction.

The freedom from protein of the materials employed has been determined by biuret and ninhydrin tests, by Kjeldahl determinations, and by removal of nitrogen by dialysis.

The colloidal cholesterol solution was prepared by dissolving the cholesterol in hot acetone and adding the solution drop by drop to a definite quantity of distilled water in constant motion. The strength of the solution used was such that 1 c c contained 0.001 gm. of cholesterol. This colloid is opaque, milk white and practically permanent. It can be filtered through paper and boiled without damage. It is electronegative and easily flocculated by bivalent and trivalent kations in proper concentrations. The particles are visible microscopically under high power and low illumination, have lively brownian movement and vary somewhat in form, but not greatly in size. The effect of adding the fat antigen, either in powder or solution, to this colloid is one of dispersion. In the proportion by weight of four parts of cholesterol to one of antigen there is little or no change save that the particles appear to become more rounded in contour. It may be said that this proportion of antigen is just sufficient for distribution at the surfaces of the phase cholesterol-water without producing alteration. Greater concentration, however, causes dispersion of the cholesterol particles accompanied by clearing and transparency of the mixture.

The first stages of this change may be seen under ordinary high power. It is not solution or saponification as the salts are at first neutral in reaction, and the change occurs almost instantly.

The typhoid suspension was used for its surface action, being a suspension of particles similar in toxifying power to agar.[2] The aqueous solution of the fatty salts, or antigen, is likewise electronegative. Interesting in this connection are the experiments of Gengou[3] on the agglutination and hemolysis of red blood cells by barium sulphate. We have found that this salt flocculates the corresponding fat antigen equally well, as it does also the fat antigens of the gonococcus-meningococcus group. Previous observations had shown that this salt and calcium chlorid are powerful agglutinants of both members of this group. With the materials enumerated it was believed that the desired effects mentioned earlier might be obtained.

Exper. 1.—Preliminary tests on rabbits weighing about 2,000 gm. were made to determine the toxicity of the 3 solutions on intravenous injection. Neither the aqueous solution of the fat antigen nor the typhoid suspension in doses suitable for the work produced harmful symptoms. The cholesterol colloid, however, proved fatal within 3 minutes following injections of 2 c c, with typical signs of anaphylaxis. Doses of 1 c c produced severe shock, but the animals recovered. The working dose adopted was 1 c c diluted with an equal volume of water. Guinea-pigs were found to be less sensitive.

Twelve rabbits averaging 1,900 gm. received the following intravenous inoculations daily for 3 days:

Rabbit 1: 5 mg. red blood cell antigen in 2 c c distilled water.

Rabbit 2: 3 c c typhoid suspension; then, after 30 minutes, 5 mg. blood cell antigen in 2 c c distilled water.

Rabbit 3: Duplicate of No. 2, interval of 15 minutes.

Rabbit 4: Cholesterol colloid, 1 mg. in 2 c c water.

Rabbit 5: Cholesterol colloid, 1 mg. in 2 c c water + 2.5 mg. blood cell antigen.

Rabbit 6: Typhoid suspension; 3 c c containing 2.5 mg. blood cell antigen.

Rabbit 7: Duplicate of No. 5.

Rabbit 8: Cholesterol colloid, 1 mg. in 2 c c water; then, after 20 minutes, blood cell antigen 5 mg. in 3 c c water.

Rabbit 9: Typhoid suspension, 3 c c.

Rabbit 10: Normal rabbit received no injections.

Rabbit 10A: Distilled water, 3 c c.

Rabbit 10B: Distilled water, 3 c c.

The animals were bled from the heart on the 13th day after the last injection, the serums separated and inactivated. In doses of 0.04 c c, in the presence of 0.02 c c of complement (60%) and 0.5 c c of 4% red blood cell suspension

[2] Novy and DeKruif: Jour. Infect. Dis., 1917, 20, p. 629.

[3] Ann. de l'Inst. Pasteur, 1904, 18, p. 678. Bordet: Studies in Immunity, collected and translated by Gay, 1909, p. 312.

in salt solution in a total volume of 1 c c, the serums produced hemolysis as follows:

Rabbits 2, 3 and 7, 100% hemolysis.

Rabbit 1, 90% hemolysis.

Rabbits 5 and 6, 50% hemolysis.

Rabbit 4, 25% hemolysis.

Rabbits 8, 9, 10, 10A, 10B, no hemolysis.

The experiment was then repeated by incubating together the rabbits' serums, the fat antigen, complement and salt solution for ½ hour at 37 C., followed by a second incubation after addition of red blood cell suspension. The hemolysis in this instance was the reverse of the preceding.

Rabbits 8, 9, 10, 10A, 10B, complete hemolysis, or no complement fixation.

Rabbit 4, partial hemolysis, or 75% complement fixation.

Rabbits 5 and 6, partial hemolysis, or 50% complement fixation.

Rabbit 1, slight hemolysis, or 90% complement fixation.

Rabbits 2, 3 and 7, no hemolysis, or complete complement fixation.

The serums were then tested for cross fixations with syphilitic, gonococcus, pneumococcus, and B. welchii antigens with negative results. With the typhoid antigen, Serum 2 gave 25% fixation, Serum 5, 10%. The pneumococcus and typhoid antigens will be referred to later. This experiment shows the presence of antibody in the serums of all but one of the animals that received the antigen.

Exper. 2.—Rabbits 1, 2, 4, 5, 7 and 8 received a second series of the same injections, commencing on the 17th day following the last of the first series and continued for 5 days.

Rabbit 3 received no injections, being reserved for control.

Rabbit 6 died in anaphylaxis immediately following the first injection of the second series.

Coincidently with these injections a fresh group of rabbits of similar average weight received the following intravenous inoculations:

Rabbit 11: Cholesterol colloid, 1 mg., and 0.25 mg. blood cell antigen in 2 c c distilled water.

Rabbit 12: Cholesterol colloid, 1 mg., and 1 mg. blood cell antigen in 2 c c water.

Rabbit 13: Cholesterol colloid, 1 mg., and 4 mg. blood cell antigen in 2 c c water.

Rabbit 14: Cholesterol colloid, 1 mg., and 8 mg. blood cell antigen in 2 c c water.

All animals were bled from the heart on the 5th day following the last injection and their inactivated serums tested as in Exper. 1.

Of the first series: Control Rabbit 3 serum showed 100% hemolysis.

Rabbits 1 and 7 serum showed 100% hemolysis.

Rabbits 2 and 8 serum showed 90% hemolysis.

Rabbits 4 and 5 serum showed 50% hemolysis.

Of the second series: Rabbits 11 and 14 serum showed 100% hemolysis.

Rabbit 13 serum showed 90% hemolysis.

Rabbit 12 serum showed 50% hemolysis.

These results were then checked and controlled by complement fixation and cross fixations as in the previous experiment, using, however, 3 antigens, one the blood cell fat antigen and two others differing slightly in strength only, having the composition noted in Exper. 3. From the results obtained it will be observed that the control rabbit had preserved its antibody strength, Rabbits 1 and 8 had gained, while all rabbits of the second series showed good antibody production.

In view of the fact that it had been possible in the case of the gonococcus to substitute an artificial product for the antigen derived from the germs,[4] it was determined to attempt an artificial substitute for the antigen of the red blood cells of the sheep. Accordingly, the total fatty acids from a large amount of thoroughly washed corpuscles were isolated and examined. The average values were as follows: Melting point, 78-80 C.; neutralization value, 140 KOH; iodin value, 70; calculated molecular weight, 400. This fat complex is somewhat unusual. The fatty acid corresponding most nearly in melting point and molecular weight was found to be cerotic acid with M. W. 396 and M. P. 78 C., but being a saturated acid has no iodin value. One apparently had to look then for a higher acid in combination with an unsaturated fatty acid of high iodin value. Such a combination, and probably the only one which would satisfy the requirements, was effected by combining melissic acid $C_{30}H_{60}O_2$, three parts, cerotic acid $C_{26}H_{52}O_2$, one part, and clupanodonic acid, $C_{18}H_{28}O_2$, an open chain acid having 8 unsaturated bonds, one part. This mixture approximates the melting point desired and has a theoretical M. W. of 405, and an iodin value of 73. Such a combination speaks for stability and high combining power. The melissic and cerotic acids were isolated from beeswax, and the clupanodonic acid was made from cod-liver oil by brominating the unsaturated fatty acids in ether, separating the octobromid and converting it into the acid by the action of zinc in HCl — alcohol, according to methods detailed by Lewkowitsch.[5] The sodium salts were then obtained from these acids and combined in the desired proportions.

Exper. 3.—Four rabbits of 2,000 gm. average weight received the following intravenous injections daily for 5 days:

Rabbit 15: Artificial blood cell antigen, 5 mg. in 2 c c water.
Rabbit 16: 3 c c typhoid suspension containing 5 mg. blood cell antigen.
Rabbit 17: Cholesterol colloid, 1 mg., and 1 mg. blood cell antigen in 2 c c water.
Rabbit 18: Cholesterol colloid, 1 mg., and 5 mg. blood cell antigen in 2 c c water.

The animals were bled 5 days after the last inoculation. Tests for the presence of antibody resulted as follows:

Rabbit 15, 100% hemolysis.
Rabbit 16, 90% hemolysis.
Rabbit 17, 75% hemolysis.
Rabbit 18, 90% hemolysis.

[4] Warden: Jour. Lab. and Clin. Med., 1916, I, p. 5; Jour. Am. Med. Assn., 1917, 68, p. 432.

[5] Chem. Technology of Oils, Fats and Waxes, 1914, I, p. 210 and p. 560 et seq.

Natural and artificial antigens gave identical readings both in hemolytic and complement fixation tests, as in the preceding experiments. There were no cross fixations with the antigens of gonococcus, treponema pallida, or pneumococcus. At this point it may be mentioned that all antigens have been controlled by fat salts derived from ordinary neutral fats such as lard, olive, cod-liver oil, coconut oil and palm oil.

From this experiment it is seen that the blood cell antigen has been approximated artificially, and that antibodies appear in the serum of rabbits injected with it.

Of the rabbits used in the preceding experiments there were selected the following for further observation, Nos. 1, 3, 8, 13, 14, and 15, these being in the best physical condition. It was observed that some had become cachectic during the process of immunization. After an interval of 14 days without injections these rabbits were again bled and the serums tested.

Rabbit 1 gave 50% hemolysis.
Rabbit 3 gave 50% hemolysis.
Rabbit 8 gave no hemolysis.
Rabbit 13 gave 100% hemolysis.
Rabbit 14 gave 75% hemolysis.
Rabbit 15 gave 75% hemolysis.

One week later, that is 3 weeks following the last inoculation, the rabbits referred to received intravenous inoculations daily for 4 days of 15 mg. artificial blood cell antigen and 2 mg. cholesterol colloid in 4 c c water. Rabbits 1 and 8 died immediately following the first of the injections. The remaining 4 animals were bled on the 5th day following the last injection and the serums tested for hemolytic power.

Rabbits Nos. 3, 13, 14, and 15, that is to say, all remaining animals, yielded serums which gave 100% hemolysis in doses of 0.04 c c of a 1 : 10 dilution. In Rabbits 13, 14, and 15 the serums possessed still higher titer, but its extent was not determined.

PRECIPITATION

Having shown that specific antibody can be produced in the serum of rabbits by injections of natural and pure artificial fat antigens in the form of sodium (or potassium) salts, it was determined to test whether such immune serums had the power to produce specific precipitation or flocculation when brought in contact with the antigen in the test tube. With the proportions by weight of antigen, cholesterol and serum employed in complement fixation as a guide the tests were undertaken first with water and salt solutions of the antigen, with such solutions in combination with aqueous cholesterol colloid, and

varying amounts of the serums to be tested. Without going into detail it was found that definite and specific precipitates were obtained with the aqueous solutions, but that it was difficult to obtain the opimum opacity of the mixtures with them and consequently difficult to get clear-cut readings. Alcoholic solution of the antigen and cholesterol was then substituted for the aqueous solutions. This alcoholic solution was the same as used for complement fixation, that is, 1 c c of the solution contained 0.002 gm. of artificial antigen and 0.008 gm. of cholesterol. With this antigen, salt solution and varying amounts of serum the tests were successful, giving complete clear-cut flocculations with settling of the precipitate and clearing of the fluid. A large number of tests were made from the data of which the following technic and protocol are given.

Into perfectly clean, small test tubes 1 by 8 cm., set in a convenient rack, there is pipetted 0.5 c c of salt solution. By means of a pipet so standardized as to drop a definite number of drops of the antigen to the gm., antigen is dropped squarely into the salt solution, the number of drops used being regulated by an optimum opacity of the resulting mixture after shaking. The mixture should be opalescent and "silky" by transmitted light and free from macroscopic particles. Too slight an opacity yields too small a precipitate while too dense an opacity may delay or prevent precipitation. From uniform pipets yielding drops of serum averaging 0.04 c c in quantity the control and determinant serums are dropped into the tubes containing the antigen and salt solution. All tubes are then thoroughly shaken. The method of dropping was found to be superior to that of allowing the substances to run down the inside of the tubes. The rack of tubes is then placed in the ice-box, but not in contact with ice, for 6-8 hours, or over night. Incubation at 37 C. in a water bath is not so satisfactory. At the expiration of this time the control tubes will show the same opacity as at the time of mixing while the positive tubes show complete flocculation, sedimentation and clearing. If the mixtures stand too long the control tubes of salt solution and antigen may show slight agglutination but without clearing or settling. It will be understood that all aqueous solutions of fat salts undergo slight changes with time owing to hydrolysis.

In the following tabulation the dose of antigen used, 6 drops, approximates $\frac{1}{16}$ gm. of the cholesterinized alcoholic antigen, or about 12 doses as employed in complement fixation, whereas the dose of serum varies from 2-4 times that used in complement fixation. In other words, the ratio of antigen to serum is about 3 times that used in the complement fixation test.

This antigen dosage represents about the maximum. With the same amount of salt solution, antigen doses of 4 drops gave equally good results but with a less amount of precipitate. The same results were obtained by using twice the quantity of salt solution and twice the

dosage of antigen and serum. When it is desirable to estimate the strength of the antibody titer 4 or more tubes may be used for each serum into which the doses of serum may be made to range from 0.02-0.32 or more.

TABLE 1

PRECIPITATIONS WITH ARTIFICIAL BLOOD CELL ANTIGEN

Each tube contains 0.5 c c salt solution and 6 drops of antigen + serum

0.12 c c salt solution	= —	0.24 c c typhoid serum	= —
0.24 c c salt solution	= —	0.12 c c normal rabbit serum	= —
0.12 c c normal human serum	= —	0.24 c c normal rabbit serum	= —
0.24 c c normal human serum	= —	0.12 c c Rabbit 3 serum	= +
0.12 c c meningococcus serum	= —	0.24 c c Rabbit 3 serum	= +
0.24 c c meningococcus serum	= —	0.12 c c Rabbit 13 serum	= ++
0.12 c c gonococcus serum	= —	0.24 c c Rabbit 13 serum	= ++
0.24 c c gonococcus serum	= —	0.12 c c Rabbit 14 serum	= ++
0.12 c c syphilitic serum	= —	0.24 c c Rabbit 14 serum	= ++
0.24 c c syphilitic serum	= —	0.12 c c Rabbit 15 serum	= ++
0.12 c c typhoid serum	= —	0.24 c c Rabbit 15 serum	= ++

The tubes were shaken after mixing and kept in the ice-box for 6 hours. The sign —, indicates no precipitation; +, partial precipitation, and ++, complete precipitation. It is not necessary to use control tubes containing salt solution and cholesterol alone because the latter is precipitated in the absence of the fat salt which tends to maintain it in high dispersion. In controlling these tests with normal human serum it was found occasionally that precipitation occurred. These serums were shown by other means to contain natural antisheep amboceptor.

THE GONOCOCCUS ANTIGEN

The gonococcus fat antigen, both natural and artificial, has been mentioned in earlier papers.[4] In order to determine whether antigens made from types of cells other than the animal red corpuscles would produce antibodies in the serums of animals injected with them the gonococcus artificial fat antigen was tested as follows:

Exper. 4.—Four rabbits, approximately 2,000 gm. in weight, received intravenous inoculations of antigen daily for 5 days.

Rabbit 19; gonococcus antigen, 8 mg. in 2 c c water.

Rabbit 20; typhoid suspension, 2 c c containing gonococcus antigen, 8 mg.

Rabbit 21; cholesterol colloid, 1 mg., and gonococcus antigen, 0.25 mg., in 2 c c water.

Rabbit 22; cholesterol colloid, 1 mg., and gonococcus antigen, 4 mg., in 2 c c water.

The rabbits, except No. 21 which died, were bled on the 5th day following the last inoculation and the serums tested.

Rabbit 19 gave 100% fixation.

Rabbit 20 gave no fixation.

Rabbit 22 gave no fixation.

For purposes of control the serums were also tested with syphilitic, pneumococcus, typhoid and red blood cell antigens with negative results. Control serums from normal rabbits, rabbits injected with cholesterol, rabbit antityphoid, normal human, syphilitic and gonococcus positive human serum were regularly negative with the gonococcus antigen except the last mentioned which was used as a positive control.

A fresh rabbit, No. 43, was substituted for Rabbit 21, and inoculations were resumed in Rabbits 20 and 22 and begun in 43. Commencing 9 days after the last previous injection each rabbit was inoculated daily for 3 days as follows:

Rabbit 19; no injection.

Rabbit 20; cholesterol colloid, 1 mg., and gonococcus antigen, 1 mg., in 2 c c water.

Rabbit 22; cholesterol colloid, 1 mg., and gonococcus antigen, 4 mg., in 2 c c water.

Rabbit 43; cholesterol colloid, 1 mg., and gonococcus antigen, 0.25 mg., in 2 c c water.

Twenty-four hours after the last injection the rabbits were bled. The serums reacted to complement fixation in the following manner:

Rabbit 19, no fixation.
Rabbit 20, 100% fixation.
Rabbit 22, 50% fixation.
Rabbit 43, 75% fixation.

Six days later, without further injections, the serum gave these results:

Rabbit 19, no fixation.
Rabbit 20, 50% fixation.
Rabbit 22, no fixation.
Rabbit 43, 50% fixation.

Two days later, 8 days after the last injection, Rabbits 20, 22 and 43 received on successive days 2 inoculations similar to those preceding. Two days thereafter their serums had changed again.

Rabbit 20, no fixation.
Rabbit 22, no fixation.
Rabbit 43, 100% fixation.

On the day following the last bleeding the same rabbits received the first of a final series of 5 daily injections of 20 mg. of antigen and 4 mg. of cholesterol in 3-4 c c of water. After an interval of 4 days the serums of Nos. 19, 22 and 43 were negative and the serum of No. 20 again gave 100% fixation. For convenience the tests are compared in the following table:

TABLE 2

COMPARATIVE SUMMARY OF A SERIES OF FIVE TESTS

Rabbit No.	Test 1	Test 2	Test 3	Test 4	Test 5
19	100%	Neg.	Neg.	Neg.	Neg.
20	Neg.	100%	50%	Neg.	100%
22	Neg.	50%	Neg.	Neg.	Neg.
43		75%	50%	100%	Neg.

These tests show that the injection of gonococcus antigen gives rise to a specific antibody in the serum of rabbits, but that the antibody is not great in amount and is transient in duration. This result is in accord with what takes place in the serum of man during the course of gonorrhea. It is well known by those experienced in the complement fixation test that the gonococcus antibody is often transient, coming and going during the progress of the disease. A negative test, therefore, may occur when gonococci are present and demon-

strable by smear and culture, and a single negative test, accordingly, may mean nothing. Repeated tests at intervals must be made before conclusions can be drawn. On the other hand, a single positive test is always of value. To illustrate: Of 58 prostitutes with gonorrhea under observation at the present time on whom single serum tests and many microscopic tests were made, 40 were positive to both smears and serum test, 12 were regarded as positive from smears alone in which the serum test was negative, and the diagnosis was established in 6 by the serum test in which smears failed.

Precipitation Test.—The precipitation test as described under the red blood cell antigen was applicable to the gonococcus fat antigen and antigonococcic serums when the latter were tested at the height of the antibody curve.

TABLE 3

SUMMARY OF RESULTS

To 0.5 c c salt solution and 4 drops of gonococcus antigen, serums were added with results as follows:

0.28 c c salt solution	= −	0.04 c c Rabbit 43 serum	= −
0.28 c c normal rabbit serum	= −	0.12 c c Rabbit 43 serum	= −
0.28 c c typhoid serum	= −	0.20 c c Rabbit 43 serum	= −
0.28 c c syphilis serum	= −	0.28 c c Rabbit 43 serum	= −
0.04 c c Rabbit 20 serum	= −	0.04 c c human serum	= −
0.12 c c Rabbit 20 serum	= +	0.12 c c human serum	= ++
0.20 c c Rabbit 20 serum	= −	0.20 c c human serum	= ++
0.28 c c Rabbit 20 serum	= ++		

PNEUMOCOCCUS FAT ANTIGEN

Having shown in the cases of the red blood cell of the sheep and of the gonococcus that specific antibodies against these cells are produced in the blood of rabbits by injecting the animals with the salts of the specific fats of the cells, similar experiments were made with the fats of the pneumococcus to determine whether they possess antigenic power.

Antigens were prepared as follows: Mass cultures of Types 1, 2, and 3, separately and combined, were grown in fat free neutral beef-peptone-glucose broth in the presence of $CaCO_3$. At first, the germs were recovered by centrifugation but the process was found to be too slow when handling 20 or 30 liters of broth. Thereafter the 24 to 48-hour growths were saponified directly in the flasks by adding to each an excess of KOH and heating to 115 C. in the autoclave for one-half hour, after which the contents of the flasks were transferred to large evaporating dishes and concentrated to one-half or more volume on water baths under an air blast. The fluid was then made distinctly acid with sulphuric acid and brought to a boiling point after which it was transferred to tall beakers and solid sodium chlorid added nearly to saturation. The beakers were then set aside, after thorough stirring of the contents, under

glass covers until all the fatty acids had separated and risen to the surface, whereafter all the liquid except the top layer was carefully siphoned off and thrown away. From this small amount of residual fluid the acids were easily separated by ether and the remainder of the process was carried through in the usual way. The sodium salts of the fatty acids obtained from the individual types and from their combination were dissolved in alcohol in the proper concentration and tested for antigenic value against known antipneumococcus serums (New York State Health Laboratory and Rockefeller Institute).

The results of the tests may be briefly summarized: Fat antigen Type 1, gave perfect complement fixation with all three types of serum.

Fat antigen Type 2 gave perfect complement fixation with serums 2 and 3, and 50% fixation with Serum 1.

Fat antigen Type 3 gave the same fixations as Type 2.

The fat antigen from the mixed types was quite unsatisfactory, giving partial fixations only with Serums 1 and 3. There was, of course, no means of knowing whether all 3 types of cocci developed equally in the culture medium but it was inferred they did not do so. The difficulties encountered in isolating these antigens, due to the large bulk of culture medium necessary, led to a study of the fats in the hope that artificial antigens might be made. These artificial substitutes have been prepared and while they are still under consideration and undergoing tests it may be permitted here to summarize the results of complement fixation and immunization with them up to the present time.

An antigen to cover the 3 types of pneumococcus has been shown to give with the control serums of the 3 types complement fixation complete and equal with each, and no fixations with normal horse serum, or with syphilitic, gonococcus or red blood cell serums. With the serums of pneumonia patients in various stages of the disease there were few positive tests, and it was apparent that even about the time of the crisis the antibody quantity is seldom large and is transient. On the other hand, a certain number of persons appear to have antibody in their blood under normal conditions.

Exper. 5.—Four rabbits of 2,000 gm. average weight received the following intravenous injections of pneumococcus antigen daily for 5 days:

Rabbit 23, pneumococcus antigen 5 mg. in 3 c c water.

Rabbit 24, pneumococcus antigen 5 mg. in 3 c c typhoid suspension.

Rabbit 25, pneumococcus antigen 0.25 mg. and cholesterol colloid, 1 mg., in 3 c c water.

Rabbit 26, pneumococcus antigen 4 mg. and cholesterol colloid, 1 mg., in 3 c c water.

The rabbits were bled on the 5th day after the last inoculation. On testing the serums it was found that Rabbits 23 and 24 gave 100% fixation of complement, Rabbits 25 and 26 partial fixation. The normal and other controls were the same as with the gonococcus and red cell serums. This group of rabbits received a second series of injections of the antigen daily for 3 days, commencing on the 9th day after the last of the previous injections. The samples of blood were drawn after the expiration of 24 hours following the last inoculation of the series. The tests showed that the serums of Rabbits 24 and 26 gave 100% fixations of complement while Rabbits 23 and 25 gave no fixation whatever. Eight days later, without further injections all serums were negative except Rabbit 26 which still gave 100% fixation.

Two days later, that is 11 days after the last injection of the second series, Rabbits 23, 25 and 26 received a third series of injections, similar to the first,

on 2 successive days, and were bled after the expiration of 48 hours. The serums of all 3 animals gave complete fixations of complement. The following table summarizes the results:

TABLE 4
Summary of Results Obtained in Exper. 5

Rabbit Number	Test 1	Test 2	Test 3	Test 4
23	100%	0	0	100%
24	100%	100%	0	Not tested
25	50%	0	0	100%
26	50%	100%	100%	100%

This experiment shows that specific antibody was produced in the blood of the animals injected with the fat antigen. It will be borne in mind that the antigen against which these tests were made was intended to be a general one and was not used as a means of identifying the strains. Only one agglutination test was made with Serums 24 and 26 controlled by normal rabbit serum, in which 0.5 c c of the serum, at the height of the antibody curve, was placed in contact with 0.5 c c of salt solution containing suspensions of Types 1, 2 and 3 from 24-hour blood-agar slants. Both serums agglutinated all 3 types in 2 hours at room temperature. As there was insufficient serum at the time for duplicate tests the procedure must be repeated.

The transient character of the pneumococcus antibody is as pronounced as in case of the gonococcus antibody. It is our observation also that the antibody in all antipneumococcic serums is not large in amount and is apt to be unstable. Those that have been kept cool and have been tested soon after inactivation react well but the antibody appears to deteriorate fairly rapidly after exposure to air, light and inactivation.

Precipitation Tests.—A series of tests were made on the precipitating properties of mixtures of pneumococcus antigen and antipneumococcus serums in salt solution in the same manner as was done with the red blood cell and gonococcus antigens and serums. The results of the tests with the general antigen and known serums are given in Table 5.

Slight alteration in the proportions of the ingredients does not interfere with the reaction as shown by using mixtures of 1 c c salt solution, 6 drops of antigen and graduated amounts of serum from 0.08-0.32 c c. The readings were practically identical. While the difference between the degrees of precipitation given by Serums 1 and 2 with the general antigen were so slight as to be detected with diffi-

TABLE 5

PRECIPITATION WITH ANTIPNEUMOCOCCUS SERUMS 1, 2, AND 3 AND PNEUMOCOCCUS ANTIGEN

In all cases 0.5 c c of salt solution and 5 drops of antigen were mixed with serum, etc.

0.04 c c salt solution..............	= —	0.16 c c pneumococcus serum 1.....	= ++
0.24 c c salt solution..............	= —	0.24 c c pneumococcus serum 1.....	= ++
0.04 c c normal horse serum........	= —	0.04 c c pneumococcus serum 2.....	= ++
0.12 c c normal horse serum........	= —	0.08 c c pneumococcus serum 2.....	= ++
0.24 c c normal horse serum........	= —	0.16 c c pneumococcus serum 2.....	= ++
0.04 c c typhoid serum............	= —	0.24 c c pneumococcus serum 2.....	= ++
0.12 c c typhoid serum............	= —	0.04 c c pneumococcus serum 3.....	= ++
0.24 c c typhoid serum............	= —	0.08 c c pneumococcus serum 3.....	= +
0.04 c c pneumococcus serum 1.....	= ++	0.16 c c pneumococcus serum 3.....	= +
0.08 c c pneumococcus serum 1.....	= ++	0.24 c c pneumococcus serum 3.....	= +

All tubes in ice-box, with occasional shaking, for 6 hours.
— indicates no precipitation; + partial, and ++ complete precipitation.

culty the precipitation with Serum 2 was the sharper and more clear-cut of the two. On the other hand, that given by Serum 3 was not so clearly defined as either of the others. This fact seemed to indicate that the antigen represented an average of slight variations in the proportions of the same fat constituents, and that by varying the proportions somewhat toward either extreme it would be possible to make antigens for Types 1, 2, and 3. This possibility had been indicated in earlier work on the fixation of complement with the antigen and the serums of the 3 types in which it was shown that variations of the same components of the fat antigen gave complete fixations with one type or another while the general type gave about equal fixations with all types. Accordingly the fatty ingredients of the antigen were combined in varying proportions above and below the average represented by the blanket type and were tested with the serums Types 1 and 3. The results showed that at certain points in these varying proportions precipitation became definite for the types and that beyond these points the reactions faded out. This showed fairly conclusively that the antigen types had been approximated. The results of the tests with Antigens 1 and 3 with the corresponding serums are shown in Table 6. Table 5 then illustrates the test of Type 2 antigen with its corresponding serum.

THE TYPHOID ANTIGEN

In a recent paper[1] it was shown that the fat complex peculiar to the typhoid bacillus gave specific complement fixation with antityphoid serum. By methods similar to those mentioned in connection with the red blood cell and other antigens a tentative artificial typhoid fat antigen of a general type has been worked out. The rather complex assortment of fats existing in the bacillus has made the antigen difficult

TABLE 6

PRECIPITATION, ANTIGEN TYPE 1 AND 3, SERUM TYPE 1 AND 3

Antigen Type 1		Antigen Type 3	
0.04 c c serum Type 1	= ++	0.04 c c serum Type 3	= ++
0.08 c c serum Type 1	= ++	0.08 c c serum Type 3	= ++
0.16 c c serum Type 1	= ++	0.16 c c serum Type 3	= ++
0.24 c c serum Type 1	= ++	0.24 c c serum Type 3	= ++
0.04 c c serum Type 3	= +	0.04 c c serum Type 1	= +
0.08 c c serum Type 3	= +	0.08 c c serum Type 1	= +
0.16 c c serum Type 3	= +	0.16 c c serum Type 1	= +
0.24 c c serum Type 3	= +	0.24 c c serum Type 1	= +
0.32 c c serum Type 3	= —		

The controls in each case are the same as in the preceding tests, and the tests were done in exactly the same manner.

to approximate, and up to the present time no effort has been made to produce separate antigens for the different antiserums of the members of the typhoid group. By means of the complement fixation test the antigen has been shown to possess group specificity for known serums of rabbits immunized with the bacilli of typhoid, paratyphoid A, paratyphoid B, and dysentery (Flexner), with the colon bacillus, and with serums of patients with typhoid fever which gave positive agglutination.

The power of this antigen to produce antibody in the blood of animals has been shown in the case of rabbits by injecting them intravenously with varying amounts of antigen in a manner similar to that used with other antigens, with the exception that suspensions of typhoid bacilli were not used, the surface action being supplied by cholesterol alone. The serums of the 4 rabbits so treated, controlled as in former tests, gave the following reactions at different periods during the course of the injections:

TABLE 7

SERUM REACTIONS OF FOUR RABBITS TREATED WITH TYPHOID ANTIGEN

Rabbit Number	Test 1	Test 2	Test 3	
4	50%	0	0	Fixation
31	100%	100%	50%	Fixation
32	0	100%	100%	Fixation
42	50%	0	0	Fixation

Rabbits 4, 31 and 32 had been used in previous experiments for purposes of control, having received injections of cholesterol colloid only. Rabbit 42 was a fresh animal.

Precipitation tests carried out as in previous experiments showed reaction to occur between the antigen and the known control antiserums of typhoid, paratyphoid A, paratyphoid B, colon and dysentery (Flexner) bacilli. Rabbit Serums 31 and 32 also gave positive reactions and produced agglutinations of suspensions of the members of the group with the exception of dysentery, which was not tried.

The facts brought out in the foregoing experiments in which it has been shown that certain fatty ingredients peculiar to four different

groups of cells give rise to specific antibody in the serums of animals appear to introduce a new phase into the problem of immunity. The work hitherto done on the relation of fats to antibody causation has dealt exclusively, so far as I am aware, with alcoholic extracts and so-called lipoids extracted by various solvents and the results have not been free from the criticism that the extractives contained protein. With the work under consideration, however, this criticism is only begging the question, and it seems to me that the pure fats must be recognized as playing a definite rôle in the immune processes. The response of the blood and cells of an animal that has been immunized with a protein in which a protective substance or state called antibody is induced has come to be recognized as definite, and to be fairly well understood in so far as the certainty of the results is concerned. This antibody is recognized by the delicate reactions of complement fixation and precipitation which occur when it is brought in contact in proper proportion with its antigen, and by certain well defined reactions, mild or fulminating, in the body of the animal. I believe we have shown that the same antibodies may be produced by fats alone and that they may be recognized by the same train of symptoms in the animal, by complement fixation, and by a delicate and specific precipitation test. It is just at this point at which the new phase referred to appears. The immunity conferred by protein alone and that produced by cells may be, and probably is, different. Protein is a constituent of cells. So is fat, but until now it seems as if the effort has been deliberately to disregard it or get it out of the way as calculated to confuse the question. Now the evidence from our experiments shows that the response to the fats is highly specific, whereas the same cannot be said for the proteins wherein specificity is confined more to type and less to species. The antibody against egg white from one source will react with egg white of another species, casein antibody responds to general casein antigens, and hemoglobin is hemoglybin from whatever source. The animal body, however, recognizes minute variations in the fat complexes such as are found in cells and is able to distinguish between related members of the same species, if not indeed individuals, as evidenced in the cases of the pneumococcus and the typhoid organisms.

In the problem the fat of the cell can be no more disregarded than its protein and should be recognized as one of the most important constituents. A study of the physiology and chemicophysics of cells leads

us to regard as fairly clearly established the function of the fats in maintaining at their surfaces a phase different from that in their interior and one instrumental in the regulation of surface energies and permeability. This matter has been discussed before,[6] and a most excellent review of the properties of cell surfaces is given by Bayliss.[7] It is well known that in certain bacteria and generally accepted that in nearly all cells the fats constitute a large but not necessarily exclusive part of their surfaces. When foreign cells invade the bodies of hosts it is precisely these surfaces which come in contact with the fluid colloids and exert influence on them, and much of the work of recent years points to the increasing importance of the effect of surface on plasma.

Proteins in aqueous solution possess surface and the same is true of fat salts in solution as shown not only by the opacity but also by their sensitiveness to the effects of electrolytes. The less soluble soaps in suspension in water produce death by anaphylaxis in rabbits quite as well as suspensions of agar or bacteria. Even the most soluble of the potassium and sodium salts of the fats develop surfaces on standing, owing to partial hydrolysis, or immediately on dilution with a large volume of water or salt solution yielding all transition stages from gel to sol and to suspensoid. It may be objected that there is no evidence to show that the salts of the fat antigens are in the same state as the fats exist in the cells. This objection can hardly be sustained in view not only of the evidence submitted here, but also of the fact that cells are rich in soaps and potassium which emulsify all other forms of fats and which are of all forms the most powerful agents in lowering surface tension. With the continued presence in the body of the host of living invading cells or with their destruction within the tissues a particular fat complex is probably continually in contact with the plasma in much the same form as in the antigens.

Each bacterial cell appears to have fat peculiar to itself which as a whole constitutes a definite complex or entity, or configuration. Chemists would call it a eutectic mixture. Group relationship in bacteria such as the types of pneumococci and the typhoid bacilli is characterized as to fats by minute variations of the same constituents, and these variations in themselves constitute definite complexes. Organisms wide apart in species as for instance the gonococcus, the

[6] Warden: Jour. Am. Med. Assn., 1917, 68, p. 432.
[7] Principles of Physiology, 1915.

tubercle bacillus, and the typhoid bacillus have fats wholly dissimilar. In the former case the differences are not so great but that the tests of complement fixation and precipitation differentiate the antigens narrowly, whereas in the latter case there are few or no cross fixations. But it has been observed that when antigens though wide apart as to configuration nevertheless contains an excess of one or more ingredients in common there is bound to be some overlapping as shown by occasional partial fixation or precipitin reactions. This has seemed to us to offer a partial explanation for certain partially positive reactions occurring with the syphilitic and other antigens otherwise unaccountable, but other conditions of which we know nothing may contribute to this phenomenon.

The plan of the antigen injections discussed in connection with the experiments in which it was sought to compare the effects of immunizing with pure antigen and with adjuvants having greater surface has led to no definite conclusions. Antibodies appeared as readily, apparently, to the one as the other, but in all the forms of injection it was impossible to avoid the factor of surface. It seems, however, that the best results were had from the combinations of antigen and cholesterol.

The question of the duration or permanence of the antigen-induced antibody has not been gone into fully, but it is apparent in the instances of the red blood cell and the typhoid bacillus that it does not persist as long in the body of the animal as that induced by immunization with the entire cell. There have been, however, during the period covered by experiments undoubted instances of sensitization as shown by anaphylactic signs more or less severe appearing in animals on reinjecting them after a considerable time was allowed to pass. This was noticed also on injecting animals and man with protein-free syphilitic antigen in experiments not yet reported.

The precipitation of mixtures of fat antigens and antiserums is regarded as a delicate and specific reaction, fully as much so as the precipitin test with protein antigen. While complement is unnecessary in such mixtures there is no question but that perfectly fresh serums give clearer reactions than those that have been inactivated or allowed to stand.

The question of total specificity in immunity it seems must include no one group of substances in the infecting cell. Rather must the antigen be inclusive of all determining substances. With the knowl-

edge that antibody to protein is persistent and represents broadly the type of the antigen, that the antibody produced by cells possesses more or less persistence together with high specificity for those cells, and that in general the antibody induced by fat antigens resembles that from the cells in all particulars, except possibly in degree of persistence, it may be inferred that the specificity of such antibody is in part or wholly due to the fats of the cell. Some bacterial cells such as the typhoid, spirochetes and others lead to long immunity with specificity confined to a group, while other cells, like the pneumococcus, gonococcus, meningococcus and streptococcus, yield at best a transient immunity but sharply defined specificity which makes it appear as though the fault apparently inherent in the family of coccaceae were attributable to the protein, especially since it has been possible to produce with fat antigen a higher antibody titer than has been met in actual disease and equally as high as that attained by injections of the cells.

CONCLUSIONS

The fat complexes characteristic of certain bacteria and other cells obtained either from the cells or assembled artificially are capable of replacing the cells themselves in the production of specific antibodies in the blood of rabbits injected with them.

The specificity of the antibodies obtained by the injection of cells probably depends in part or wholly on the configuration of the fats constituting the bulk of the cell surfaces.

THE COAGULATION OF THE BLOOD AND ANAPHYLACTIC SHOCK

HAROLD A. BULGER

From the Department of Physiology, Harvard Medical School, Boston

The phenomenon of anaphylaxis has received a vast amount of study in recent years, but in spite of this we are still far from an adequate explanation of its mechanism. The relation of anaphylaxis to infection, immunity reactions, specific and nonspecific treatment of infections, asthma, skin diseases, food idiosyncrasy and blood transfusion has made it a very important problem in medicine. The relation of anaphylaxis to the coagulation of the blood has also attracted some attention since anaphylactic shock is accompanied by a more or less marked change in the coagulability of the blood. These changes in coagulability have led to interesting ideas concerning the theory of anaphylaxis. The antigen-antibody reaction has been supposed by Doerr[1] to influence the process of coagulation, and he, with others, believes that these changes in coagulation are the direct cause of anaphylactic shock. The coagulation process may cause blood or serum to become toxic for the homologous animal. Schultz[2] has noted that fresh uncoagulated arterial blood has no influence on smooth muscle of the same animal, but as soon as there is evidence of clotting the muscle contracts. These phenomena are interesting and worthy of attention and probably have an important relationship to anaphylaxis. Biedl and Kraus[3] believe the decreased coagulability in anaphylactic shock to be due to a decrease in thromboplastin or to an excess of antithrombin. Lee and Vincent[5] "attribute the alteration of coagulation in anaphylaxis to a definite effect on the blood platelets and not to the introduction of antithrombin." Novy and DeKruif[6] assume that "fibrinogen is transformed into an incoagulable tautomeric

Received for publication June 8, 1918.

[1] Wien. klin. Wchnschr., 1912, 25, p. 339.
[2] Bull. 80, Hyg. Lab., U. S. Pub. Health and Mar. Hosp. Serv., 1912.
[3] Wien. klin. Wchnschr., 1909, 22, p. 363.
[4] Jour. Infect. Dis., 1914, 14, p. 476.
[5] Jour. Med. Research, 1915, 27, p. 445.
[6] Jour.. Am. Med. Assn., 1917, 68, p. 1527.

modification." Shattuck[7] found a delayed "prothrombin time," but inconclusive results with antithrombin. His antithrombin tests were made by the method of Hess.[8]

METHODS

The methods used in examining the blood in these experiments were first described by Howell[9] and further discussed by Drinker and Hurwitz[10] and by Minot, Denny and Davis.[11]

The so-called "prothrombin time" is the time required for oxalated plasma to clot after the addition of an optimum amount of calcium. Variations in prothrombin time should not be interpreted, as is often done, to mean changes in the amount of prothrombin only. Variations in antithrombin may influence this time; variations in the amount of thromboplastin may cause marked changes in the prothrombin time. The addition of thromboplastin will decrease the time greatly, while absence of thromboplastin will prevent coagulation entirely. This influence of variations in thromboplastin on the prothrombin time may be very important.

The antithrombin determinations consisted in determining the inhibitory influence exerted by oxalated plasma heated to 60 C. on the action of thrombin on fibrinogen. One drop of heated oxalated plasma was added to 2, 3, 4, and 5 drops of thrombin solution and the mixtures allowed to stand 15 minutes. At the end of this time 7 drops of fibrinogen solution were added to each tube and the clotting time determined. The amount of antithrombin in each specimen was indicated by the average clotting time of the 4 tubes. Comparisons between the antithrombin titer in different experiments cannot be made because different solutions of thrombin and of fibrinogen were used in each experiment. Here again thromboplastin may produce variations, a large amount decreasing the inhibitory influence of antithrombin, a small amount having the reverse effect.

The thrombin used in these determinations was prepared by the method of Howell,[12] and some difficulty, traced finally to poor fibrinogen, was encountered in obtaining a satisfactory thrombin-fibrinogen combination. Solutions of purified fibrinogen were found to be more satisfactory than dried plasma and gave a better end point. Fibrinogen from cat's blood proved better than that from dog's and was prepared from carotid blood collected in vaselined centrifuge tubes containing sodium oxalate solution. The plasma was procured by centrifugalization and pure fibrinogen obtained by Howell's[13] modification of Hammarsten's method. A fresh solution was prepared about every 5 days.

Anesthesia was produced by 1.5 gm. of urethan per kg. of body weight. This was found to be very satisfactory, respiratory paralysis never being encountered. In a few experiments 1.7 c c of paraldehyd per kg. was used.

Egg white dissolved in an equal volume of salt solution was employed to produce anaphylaxis.

After the animal was anesthetized, a vein for injection was exposed—the external jugular or the femoral. An artery—the carotid or in a few cases the

7 Arch. Int. Med., 1917, 20, p. 167.
8 Jour. Exper. Med., 1915, 21, p. 338.
9 Arch. Int. Med., 1914, 13, p. 76.
10 Ibid., 1915, 15, p. 733.
11 Ibid., 1916, 17, p. 101.
12 Am. Jour. Physiol., 1913, 32, p. 264.
13 Ibid., 1910, 26, p. 461.

femoral—to supply the specimens was next exposed, the distal portion ligated and the proximal portion clamped. Care was taken not to crush or injure the vessel. Blood pressure and respiratory tracings were made in a number of the experiments. The specimens of blood were obtained through vaselined glass cannulas. In order to use a fresh, clean cannula for every specimen of blood, each cannula was inserted into the artery and held in place without tying. At each collection of blood two specimens were taken. One—used to determine the coagulation time—was placed at once in a water bath at 37-38 C., and the time noted at which the tube could first be inverted without dislodging the clot. The other was the oxalated specimen. This was drawn directly into a vaselined centrifuge tube containing 1 part of 1% sodium oxalate in physiologic salt solution to 8 parts of blood. After obtaining a normal control specimen the injection was made and the vein clamped off. A second specimen was usually taken as soon after the injection as possible and other specimens followed at varying intervals.

After the death of the animal the oxalated specimens were centrifuged at high speed for 15 minutes and the plasma obtained. Determinations of "prothrombin time" and of antithrombin were made in most cases at once and never later than 3 or 4 hours after collection of the blood. Common glass pipets with rubber bulbs were used in these determinations and when comparable results were wanted the same pipet was used for all specimens, being cleaned and dried before changing from one specimen to another.

The tubes containing the "coagulation time" specimens were set aside and observed from time to time to note the rate of fibrinolysis.

Experiments with Cats

Cats were sensitized by 3 injections at 2-day intervals of 2 c c of egg white dissolved in an equal volume of physiologic salt solution. Shock was produced by intravenous injection of 5 c c of 50% egg white, 7-10 weeks later. In only one were distinct symptoms of shock with death produced in 10 minutes. Blood pressure tracings in 4 experiments showed the characteristic fall in blood pressure, but instead of remaining down this was recovered from at once. In only the one case mentioned was an incoagulable blood obtained. An average of the other cases showed a slight increase in the coagulation time preceded by a slight decrease. The antithrombin changed very little, the average determinations showing a very slight decrease in amount which might be due to the hemorrhage. The average of the prothrombin times showed a small increase preceded by a slight decrease (Table 1). After shock the plasma showed evidence of hemolysis and there was often a decrease in the "buffy coat."

Experiments with Rabbits

Rabbits were sensitized by 2 or 3 injections at 2-day intervals of 2 c c of 50% egg white solution. Shock was produced 4-8 weeks later by intravenous injection of 2-6 c c of 50% egg white solution. The results are shown in Table 1. In Exper. 1 there was no increase in coagulation time. In Expers. 8 and 9 the increase was only slight. In the remaining 5 there was a marked increase. In 4 experiments the first specimen taken after shock showed a decreased coagulation time. The average of the antithrombin determinations show a moderate decrease in antithrombin. There was an increase in prothrombin time.

The clotting time of the oxalated specimens was also determined after the addition of optimum amounts of thromboplastin and calcium. By this proce-

TABLE 1

THE EFFECT OF ANAPHYLACTIC SHOCK ON THE COAGULATION TIME AND THE FACTORS OF COAGULATION OF THE BLOOD OF CATS, RABBITS, AND DOGS

	Coagulation Time, Minutes						
	1	2	3	4	5	6	7
Cat 1	6	3½	7	7½	7		
2	7	3½	9½	7	6	4	
3	3	4	4	4			
4	3	3	(No clot)				
5	7	3	12	10	9		
6	6½	4	14	11	10	11	
Rabbit 1	6	6	3	6	6		
3	5½	26	30	29	25	17	
4	16	17	15	10	30	26	
5	12	16	51	36	32		
6	12	7½	15	60	45	59	30
7	8	23	43	53	95		
8	12	6	16	14	13		
9	8½	19	9½	11	15	14	
Dog 4	6	6	(Jelly clot in 48 hrs.)				
5	8		(Jelly clot in 48 hrs.)				

	Time after Shock, Minutes						
	1	2	3	4	5	6	7
Cat 1	*	2½	5	15	26		
2	*	3	13	25	26	29	
3	*	3½	15	24			
4	*	1½	8				
5	*	2	11	18	45		
6	*	1	7	13	42	63	
Rabbit 1	*	*	3	13	28		
3	*	2	8	13	23	35	
4	*	*	*	10	21	37	
5	*	1	12½	20	26		
6	*	2	3	7½	9	10½	13½
7	*	1½	5	10	20		
8	*	1½	10	20	30		
9	*	1½	1	13	33	60	
Dog 4	*	1½	2	6	28	63	72
5	*	5	30	60	90	120	

	Antithrombin, Minutes						
	1	2	3	4	5	6	7
Cat 1	65	62	69	66	58		
2							
3	42	42	41	39			
4	36	19	46				
5	22	20	22	20	18		
6	27	25	31	28	24	22	
Rabbit 1	68	68	52		44		
3	117	109	77	82	31		
4	23	23	23	25	19	14	
5	63	47	11	33	28		
6	111	99	87	72	66	63	61
7	48	66	49	58	29		
8	21	25	19	15	15		
9	43	39	30	31	24	27	
Dog 4	21	25	31	31	30	21	18
5	32	48	45	48	36	32	

	"Prothrombin Time," Minutes						
	1	2	3	4	5	6	7
Cat 1	2½	2½	3	3	3		
2	3	2½	3	3	3		
3	3½	3½	3½	3½			
4	3	2½	7				
5	4½	3½	6½	5½	6½		
6	3½	3	6	5½	4½	4	
Rabbit 1	1½	1½	1½		3½		
3	2½	5	5½	5½	4		
4	10	11	11	6	15	14	
5	7½	9	17	14	13		
6	10	5	6½	12	20	16	15
7	11	13½	17	23	13		
8	10	6	15	14	12		
9	35	60	50	19	13	11	
Dog 4	2	4½	5½	13½	6½	4½	3
5	2	(no clot)			15	13	

	Clotting Time of Oxalated Plasma Optimum Ca and Thromboplastin, Minutes						
	1	2	3	4	5	6	7
Cat 1							
2							
3							
4							
5							
6							
Rabbit 1							
3	2½	2½	2½	2½	2½	2½	
4	2	2	2	1¾	2		
5	1¾	1¾	1¼	1½	1¼	1¼	1
6	1¾	1¾	1½	1¾	1¾		
7	2½	2½	2½	2½	2½		
8	1½	1½	1½	1½	1½		
9							
Dog 4	1	1	1¾	1½	1½	1¼	1
5	1	2½	3	2½	2	2	

TABLE 3

THE EFFECT OF INTRAVENOUS INJECTION OF SEROTOXIN AND PEPTONE ON THE COAGULATION TIME AND FACTORS OF COAGULATION OF THE BLOOD OF RABBITS

		Coagulation Time, Minutes							
		1	2	3	4	5	6	7	8
Rabbit 13	Serotoxin.	8	11	15	14				
15	Serotoxin..	3	3½	6½	9½	8			
14	Peptone...	11	9	6	9	15	15	14	12
16	Peptone...	5	12	6	10	12			
17	Peptone...	11	16	10½	19				

		Time after Shock, Minutes							
		1	2	3	4	5	6	7	8
Rabbit 13	Serotoxin.	*	6	18	25				
15	Serotoxin..	*	3½	8½	9½	20			
14	Peptone...	*	½	6	16	40	60	90	126
16	Peptone...	*	1½	10	24	39			
17	Peptone...	*	1	10	16				

		Antithrombin, Minutes					
		1	2	3	4	5	6
Rabbit 13	Serotoxin.	72	68	72	59		
15	Serotoxin..	54	52	50	47	38	
14	Peptone...	44	43	38	37	33	26
16	Peptone...	30	28	25	20		
17	Peptone...	39	32	31	34		

		"Prothrombin Time," Minutes					
		1	2	3	4	5	6
Rabbit 13	Serotoxin.	8	12	16½	14		
15	Serotoxin..	4½	4	16	13	12	
14	Peptone...	6½	17	11	14	14	14
16	Peptone...	3½	6	4½	6		
17	Peptone...	3	6½	3—	8½		

		Clotting Time of Oxalated Plasma Optimum Ca and Thromboplastin, Minutes						
		1	2	3	4	5	6	7
Rabbit 13	Serotoxin.							
15	Serotoxin..							
14	Peptone...	2	2½	2¼	2½	2	2	
16	Peptone...	2	2½	2	2½			
17	Peptone...	1¼	2	1½	1¼			

* Control specimens taken before shock.
The figures in these tables for antithrombin indicate the average clotting time in minutes of four thrombin-antithrombin-fibrinogen mixtures, using 2, 3, 4 and 5 drops of thrombin.

dure it was intended to show whether the changes in the prothrombin time were due to variations in prothrombin or in thromboplastin. The antithrombin remaining constant one would expect to get variations in the clotting time with variations in prothrombin, or if the prothrombin remained constant to get the same clotting time in all specimens. Any variations in antithrombin would cause a change. Solutions of thromboplastin prepared by the method of Howell[14] were used. These were found to be more satisfactory than purified cephalin prepared by McLean's method.[15] All determinations were made at 37-38 C. To 5 drop portions of the oxalated specimens were added 1, 2, 3, and 4, or 1, 3, and 5 drops of the thromboplastin solution. After standing a few minutes the optimum amount of calcium chlorid solution was added to each tube and the clotting time carefully determined. The results are shown in the last column of Table 1. In all experiments the clotting time was practically the same in all specimens indicating a constant amount of prothrombin.

There was slight hemolysis in the plasma after shock and as a rule a decrease in the "buffy coat."

The following protocol illustrates a typical experiment.

Rabbit 5: Sensitized by intraperitoneal injections of 2 c c of 50% egg white on Oct. 3, 1917, and on Oct. 5, 1917.

Nov. 12, 1917: weight 2.5 kg.

4:18 p. m.: 4 gm. of urethan.
4:46½ p. m.: Control Specimen 1. Coagulation time 12 minutes.
5:01½ p. m.: 2 c c of 50% egg white injected into femoral vein.
5:02½ p. m.: Specimen 2. Coagulation time 16 minutes.
5:14 p. m.: Specimen 3. Coagulation time 51 minutes.
5:22 p. m.: Specimen 4. Coagulation time 36 minutes.
5:28 p. m.: Specimen 5. Coagulation time 32 minutes.

Plasma after shock showed slight hemolysis.

ANTITHROMBIN DETERMINATIONS

Thrombin, Drops	Antithrombin, Drops	Time Interval, Minutes	Fibrinogen, Drops	Coagulation, Minutes
		Specimen 1 (Normal)		
2	1	15	7	95
3	1	15	7	70
4	1	15	7	55
5	1	15	7	34
				Average = 63
		Specimen 2		
2	1	15	7	74
3	1	15	7	54
4	1	15	7	37
5	1	15	7	24
				Average = 47
				Per cent. of normal average = 74
		Specimen 3		
2	1	15	7	73
3	1	15	7	51
4	1	15	7	32
5	1	15	7	22
				Average = 44
				Per cent. of normal average = 70

[14] Am. Jour. Physiol., 1912, 31, p. 1.
[15] Ibid., 1916, 41, p. 250.

Specimen 4

2	1	15	7	52
3	1	15	7	37
4	1	15	7	26
5	1	15	7	16
				Average = 33
				Per cent. of normal average = 52

Specimen 5

2	1	15	7	48
3	1	15	7	31
4	1	15	7	21
5	1	15	7	13
				Average = 28
				Per cent. of normal average = 44

Control

Thrombin, Drops	Fibrinogen, Drops	Coagulation, Minutes
3	7	3½
5	7	2½

PROTHROMBIN TIME

Specimen 1 (Normal)

Oxalated Plasma Drops	0.5% $CaCl_2$ Drops	Coagulation, Minutes
5	2	15
5	3	7½
5	4	8
5	5	11
		Optimum 7½ Minutes
	Specimen 2	
5	2	9½
5	3	9
5	4	11½
5	5	12
		Optimum 9 Minutes
	Specimen 3	
5	2	25
5	3	17
5	4	24
5	5	22
		Optimum 17 Minutes
	Specimen 4	
5	2	23
5	3	14
5	4	21
5	5	19
		Optimum 14 Minutes
	Specimen 5	
5	2	13
5	3	13½
5	4	14
5	5	25
		Optimum 13 Minutes

Clotting time of oxalated plasma on addition of an optimum amount of thromboplastin and calcium:

Specimen 1 (Normal)

Oxalated Plasma, Drops	Salt Solution, Drops	Thromboplastin Solution, Drops	0.5% $CaCl_2$ Solution, Drops	Clotting Time, Minutes
5	3	1	3	2
5	2	2	3	2
5	1	3	3	3
5	0	4	3	3¼
			Optimum = 2 minutes	
		Specimen 2		
5	3	1	3	2¼
5	2	2	3	2
5	1	3	3	2½
5	0	4	3	3
			Optimum = 2 minutes	
		Specimen 3		
5	3	1	3	2
5	2	2	3	2
5	1	3	3	2½
5	0	4	3	3
			Optimum = 2 minutes	
		Specimen 4		
5	3	1	3	2
5	2	2	3	1¾
5	1	3	3	2
5	0	4	3	3
			Optimum = 1¾ minutes	
		Specimen 5		
5	3	1	3	2¼
5	2	2	3	2½
5	1	3	3	2
5	0	4	3	3
			Optimum = 2 minutes	

Experiments with Guinea-Pigs

Guinea-pigs were sensitized by one intraperitoneal injection of 1 c c of 1% egg white solution. Shock was produced by an intravenous injection of 1 c c of 50% egg white. It was not practical to collect the specimens through cannulas because the blood flowed too slowly. Therefore, with the animal under urethan anesthesia, the thorax was quickly opened, and while the heart was held with small forceps a cut was made into a ventricle with scissors and a vaselined 5 c c pipet quickly inserted into the heart. The blood was sucked up into the pipet and transferred to the tube for determining the coagulation time and to the tube containing the sodium oxalate solution. For control specimens normal guinea-pigs were used. Such controls would be of questionable value had the changes been less marked. The results are shown in Table 2. In two cases there was no change in the coagulation time. In the others there was an increased coagulation time. Two specimens were incoagulable and one clotted only after 24 hours. Averages showed slightly less antithrombin in the shocked animals than in the normal. The "prothrombin time" was increased.

Experiments with Dogs

Dog 4 was sensitized by intraperitoneal injections of 5 c c, 5.5 c c, and 5.5 c c of 50% egg white solution on Nov. 12, 14, and 18. On Dec. 27 shock

was produced by an intravenous injection of 20 c c of 50% egg white. The specimen taken one-half minute after shock showed no change in the coagulation time. The other specimens formed a jelly clot after 48 hours. Antithrombin was slightly increased. Prothrombin time was increased. By adding optimum amounts of thromboplastin and calcium to the oxalated plasma a clotting time was obtained which was nearly constant, varying slightly as the antithrombin carried and indicating a constant prothrombin content.

TABLE 2

THE EFFECT OF ANAPHYLACTIC SHOCK ON THE COAGULATION TIME AND THE FACTORS OF COAGULATION OF BLOOD OF GUINEA-PIGS

Normal guinea-pigs were used for controls. The figures for antithrombin indicate the average clotting time in minutes of 4 thrombin-antithrombin-fibrinogen mixtures, using 2, 3, 4, and 5 drops of thrombin.

Guinea-Pigs	Time after Injection, Minutes	Coagulation Time, Minutes	Anti-thrombin, Average	Pro-thrombin, Minutes
12 Normal		3	104	2½
1 Anaphylactic	4	13	74	5
13 Normal		3½	63	3
2 Anaphylactic	1½	9	56	4½
3 Anaphylactic	3	3	74	2½
4 Anaphylactic	6	24 hrs.	51	6
14 Normal		5	67	2
5 Anaphylactic	2	No clot 24 hrs.	50	5
6 Anaphylactic	2	No clot 24 hrs.	46	5½
15 Normal		3½	53	2
7 Anaphylactic	10	3	30	2
8 Anaphylactic	2	12	21	3

Dog 5 was sensitized by intraperitoneal injection of 5 c c, 10 c c, and 10 c c of 50% egg white solution on Nov. 12, 14, and 17. Shock was produced on Dec. 29 by an intravenous injection of 45 c c of 50% egg white. After shock the blood showed a jelly clot in 48 hours. Antithrombin showed a slight increase. The prothrombin time was increased. In specimens 2, 3, and 4 the oxalated plasma failed to clot on addition of calcium chlorid solution. By addition to optimum amounts of thromboplastin and calcium to the oxalated plasma the clotting time varied somewhat as the antithrombin. The results are shown in Table 1.

"SEROTOXIN"

"Serotoxin" was prepared as described by Jobling and Petersen[16] by extracting dog serum with chloroform. In one rabbit (Rabbit 10) death occurred in less than 1 minute after injection of 1 c c. The animal was examined immediately. The heart and some of the large vessels contained solid clots. The coagulation time of the blood before injection was 8 minutes while these clots were found in less than 4 minutes after the injection.

In Rabbits 11 and 12, 0.5 c c of serotoxin was injected. The procedure was the same as in other experiments. The coagulation time, prothrombin time, and antithrombin changed in the same way as in anaphylactic shock. The results are shown in Table 3. After serotoxin injection there was marked hemolysis, greater than ever produced by anaphylactic shock. No buffy coat was present.

[16] Jour. Exper. Med., 1914, 19, p. 480.

KAOLIN SHOCK

A kaolin suspension in salt solution was prepared and allowed to settle a few minutes. The top layer was removed and filtered several times. When 2 c c of this suspension was injected into rabbits intravenously it produced almost immediate death. The animals were examined at once. Almost complete intravascular coagulation had occurred in a very few minutes.

PEPTONE SHOCK

The procedure followed in the foregoing experiments was repeated with 3 rabbits, peptone solution being injected intravenously. The results are shown in Table 3. The coagulation time after initial variations was increased. The prothrombin time was increased. There was no increase in antithrombin, but a slight decrease in all cases. There was no hemolysis in the plasma after peptone injection.

FIBRINOLYSIS

The specimens used for determining coagulation times were preserved and observations made on the rate of fibrinolysis. In normal specimens the average time for solution of the clot was 30-40 days or more. In most cases there was a marked increase in the rate of solution after anaphylactic and peptone shock. In a few cases solution was complete in 4 or 5 days. Jobling, Petersen and Eggstein have shown that during anaphylactic shock[17] and peptone shock[18] there is a mobilization of serum protease and a decrease in antitryptic titer. The antitryptic power of serum has been shown by Jobling and Petersen[19] to be removed by chloroform and ether and to be due to compounds of the unsaturated fatty acids. Antitrypsin obtained by the method of Jobling and Petersen was found to possess no antithrombic activity but did possess some thromboplastic action, probably due to contamination. Minot[20] has shown that chloroform and ether render antithrombin inactive but he was unable to recover any antithrombin from such extracts.

DISCUSSION

The antithrombin changes in these experiments were not great. With dogs the antithrombin was increased somewhat. With other animals there was, as a rule, a slight but definite decrease, although the coagulation time and the prothrombin time were increased. This decrease in antithrombin may have been due to the hemorrhage, Drinker and Drinker[21] having shown that hemorrhage produces a decreased coagulation time accompanied by a decrease in antithrombin. The increased coagulation time after anaphylactic shock is therefore not due to an increase in antithrombin, although it may be possible to have an increase in some animals which would help to retard coagulation.

[17] Jour. Exper. Med., 1915, 22, p. 401.
[18] Ibid., 1915, 22, p. 597.
[19] Ibid., 1914, 19, p. 459.
[20] Am. Jour. Physiol., 1915, 39, p. 131.
[21] Ibid., 1915, 36, p. 305.

The clotting time on addition of an optimum amount of calcium chlorid to the oxalated plasma was increased after shock, and varied as the coagulation time of the blood varied. When there was a decreased coagulation immediately following shock there was a corresponding decrease in the prothrombin time. Such variations might be caused by changes in prothrombin or by changes in thromboplastin. But since the same clotting time was obtained in both normal and in shock oxalated plasma when optimum amounts of thromboplastin and calcium were added there is probably no variation in prothrombin which would account for the changes in coagulability. This fact indicates changes in thromboplastin rather than in prothrombin. Barrat[22] believes that prothrombin has little influence on coagulation time, that with a sufficient concentration of prothrombin a given amount of thromboplastin will produce a definite quantity of thrombin independent of the actual concentration of prothrombin.

There has been an increasing interest in the rôle which lipoids play in immunologic reactions, and it is probable that lipoid changes play a very important part in anaphylactic shock. Howell[14] has shown that an important thromboplastic substance of the tissues is the lipoid cephalin and McLean[23] has shown that the thromboplastic activity of cephalin is directly proportional to its degree of unsaturation. Howell believes that the active substance of the tissues is probably a cephalin-protein combination. Dale and Walpole[24] have shown that when plasma or serum is treated with chloroform or trypsin a powerful thromboplastic activity is quickly produced. This is of interest in relation to the intravascular coagulation produced by serotoxin and kaolin and the increased coagulability sometimes found just after shock. These facts seem to indicate an immediate mobilization of thromboplastin. Tissue extracts when injected into animals produce the same characteristic symptoms seen in anaphylaxis. If there is a primary mobilization of thromboplastin it is rapidly neutralized or removed. Antithrombin will neutralize thromboplastin to a certain extent. If such a reaction takes place under these conditions, variations in the stability of the combination formed in different species of animals might determine whether or not there would be an increase in free antithrombin in the blood, or some animals may respond with a greater production of antithrombin.

[22] Biochem. Jour., 1915, 9, p. 511.
[23] Am. Jour. Physiol., 1917, 43, p. 586.
[24] Biochem. Jour., 1916, p. 10, p. 331.

These phenomena seem to be further indications for more extensive studies of the lipoids of the blood.

CONCLUSIONS

The changes in the coagulability of the blood during anaphylactic shock are due to changes in that stage of the coagulation process at which thrombin is formed through the interaction of prothrombin, calcium, thromboplastin and antithrombin (?). These changes are probably due to variations in thromboplastin.

Antithrombin changes are not great. In some animals there may be an increase in antithrombin which would aid in retarding the coagulation of the blood. There is no increase in antithrombin in rabbits.

There was a marked increase in the rate of fibrinolysis after anaphylactic and peptone shock.

THE BARTLETT AND O'SHANSKY MODIFICATION OF THE WASSERMANN REACTION

E. H. Ruediger

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

Smith and MacNeal,[1] Bohan and Lynch,[2] Ottenberg[3] and others emphasize the importance of making the Wassermann reaction a more sensitive test than it was originally, and Bartlett and O'Shansky[4] propose a rapid method of doing the Wassermann reaction.

Since a reliable rapid method would be greatly appreciated by all who have to do these tests, this rapid method was compared with the method regularly used at the Bismarck Hospital.

Technic of Our Regular Method

The human serum to be tested is heated to about 56 C. for 30 minutes. All ingredients are so prepared that the test doses are uniform, as 0.2 c c of human serum, 0.2 c c of diluted complement, 0.2 c c of diluted antigen, 0.2 c c of diluted amboceptor, 0.2 c c of corpuscle suspension, in all 1 c c per test tube.

As complement serum the mixed serums of 3 or more guinea-pigs are used in dilutions of 1 : 5, 1 : 10 and 1 : 20.

The antigen is an alcoholic extract of human heart muscle; it is used in the largest quantity that is no longer anticomplementary and is diluted so that the test dose is contained in 0.2 c c.

Human blood corpuscles from nonsyphilitic persons are well washed and are made up in a 2.5% suspension in salt solution. The test dose is 0.2 c c.

As hemolytic amboceptor the serum of a rabbit which has been immunized against human blood corpuscles is used. It is diluted with salt solution so that the test dose (1 unit) is contained in the 0.2 c c put into each test tube.

The tests are quantitative up to what I at the present time call 10 +. Six test tubes are used for each test, they are designated as 1, 2, 3, 1′, 2′ and 3′. Tubes 1, 2 and 3 are antigen tubes and Tubes 1′, 2′ and 3′ are corresponding control tubes. Each of the 6 tubes receives 0.2 c c of serum to be tested Each of the first pair of tubes, Tubes 1 and 1′ receives 0.2 c c of 1 : 5 dilution of complement; each of the second pair of tubes, Tubes 2 and 2′, receives 0.2 c c of 1 : 10 dilution of complement, and each of the third pair of tubes, Tubes 3 and 3′, receives 0.2 c c of 1 : 20 dilution of complement. Each of the antigen tubes receives 0.2 c c of diluted antigen, and each of the control tubes receives 0.2 c c of salt solution. The serum-complement-antigen mixtures are put into the refrigerator for 5 hours when 0.4 c c of sensitized blood corpuscles representing 0.2 c c of corpuscle suspension and 0.2 c c of diluted hemolytic amboceptor, are put into each test tube, the tubes well shaken and put into the incubator at 37 C. for 1 hour during which they are shaken at intervals of 15

Received for publication June 10, 1918.

[1] Jour. Immunol., 1916, 2, p. 75; Jour. Infect. Dis., 1917, 21, p. 233.
[2] Jour. Am. Med. Assn., 1917, 69, p. 1220.
[3] Arch. Int. Med., 1917, 19, p. 457.
[4] Jour. Lab. and Clin. Med., 1817, 3, p. 118.

minutes. After 1 hour in the incubator they are allowed to stand at room temperature for 1 or 2 hours before the results are read and recorded. In reading and recording results, a difference of less than one-fourth of 1 tube is called faintly positive, ±; a difference of from one-fourth to one-half of 1 tube is called weakly positive, 1 +; a difference of more than one-half but less than a complete tube is called moderately positive, 2 +; a difference of 1 complete tube is called strongly positive, 3 +; a difference of 2 complete tubes is called strongly positive, 6 +; a difference of 3 complete tubes would be called strongly positive 12 +. I always adjust the hemolytic system so as to get incomplete hemolysis in the last control tube, Tube 3′. With complete inhibition in all 3 antigen tubes, complete hemolysis in Control Tubes 1′ and 2′, and nearly complete hemolysis in Tube 3′, we have a difference of about 2.75 tubes which is called strongly positive, 10 +.

If a serum tests more than 10 + and a complete quantitative test is desired more antigen tubes are placed to the left of Tube 1. These tubes are designated as d 4 (d stands for dilution), d 20, d 100, and d 500, as is shown in Table 1.

TABLE 1

Showing the Arrangement of Tubes Used in the Preliminary Quantitative Test

No. of Serum	Readings*										Results
	Antigen Tubes							Control Tubes			
	d500	d100	d20	d4	1	2	3	1′	2′	3′	
1	+	+	+	+	0	0	0	+	+	±	Strongly positive, 10+
2	+	+	+	0	0	0	0	+	+	±	Strongly positive, 40+
3	+	+	0	0	0	0	0	+	+	±	Strongly positive, 200+
4	+	0	0	0	0	0	0	+	+	±	Strongly positive, 1,000+
5	0	0	0	0	0	0	0	+	+	±	Strongly positive, 5,000+

* Explanation: 0 = no hemolysis; ± = hemolysis between 50 and 100%; + = complete hemolysis.

Table 1 shows the arrangement of tubes used in the preliminary quantitative test. Ten test tubes are used. Tubes 1, 2, 3, 1′, 2′ and 3′ each receive undiluted human serum or serum-glycerol mixture; Tube d 4 receives a 1:4 dilution of serum, Tube d 20 receives serum diluted 1:20, Tube d 100 receives serum diluted 1:100 and Tube d 500 receives serum diluted 1:500. Tubes d 4 d 20, d 100 and d 500 receive the same quantity of complement as do Tubes 1 and 1′. Antigen blood corpuscles and hemolytic amboceptor are uniform throughout.

In the tests now reported all serums which tested 10 + or more were tested twice, a preliminary titration in accordance with the scheme shown in Table 1 determined approximately the dilution to be used, then the serum was diluted, the diluted serum was tested by the regular 6-tube method and the result thus obtained was multiplied by the dilution.

Serum 2 in Table 1 shows 40 + in the preliminary titration, a dilution of 1:10 could be considered safe to use in the final titration and 5 + might be the result obtained with the diluted serum. Multiplying the result obtained, 5 +, by the dilution, 10, would give 50 + as the final result.

Technic of the Bartlett and O'Shansky Modification

The hemolytic index was not determined before the test proper was done. Ten test tubes, 5 antigen tubes and 5 control tubes were used for each test. Each of the first pair of tubes received 0.4 c c of fresh human serum; each tube of the second pair received 0.4 c c of a 1:2 dilution of fresh human serum; of the third pair of tubes each received 0.4 c c of a 1:4 dilution of fresh human serum; each tube of the fourth pair received 0.4 c c of a 1:8 dilution of fresh human serum and each tube of the fifth pair received 0.4 c c of a 1:16 dilution of fresh human serum. Each antigen tube received 0.2 c c of diluted antigen; each control tube received 0.2 c c of salt solution and to each of the 10 tubes 0.4 c c of a 2.5% suspension of sheep blood corpuscles was added. The tubes were placed in the water-bath at 37 C. for 30 minutes, after which the results were read. Only 6 tubes are recorded in the table, these are the last control tubes showing distinct hemolysis, 2 preceding control tubes and the corresponding antigen tubes.

THE COMPARATIVE TEST

Ten fresh serums from syphilitics under treatment were subjected to a comparative test.

TABLE 2

Bartlett and O'Shansky Modification of the Wassermann Reaction Compared with our Regular Method

No. of Serum	Method	Dilution of Serum	Readings* Antigen Tubes 1	2	3	Control Tubes 1′	2′	3′	Results	
1	Regular	0	tr	0	0	+	+	±	Strongly positive,	8+
	B. and O'S.		+	±	tr	+	±	tr	Negative,	—
2	Regular	1:25	+	0	0	+	+	±	Strongly positive, 5+ × 25 = 125+	
	B. and O'S.		tr	0	0	+	+	tr	Strongly positive	
3	Regular	0	±	0	0	+	+	tr	Strongly positive,	5+
	B. and O'S.		+	+	tr	+	+	tr	Negative,	—
4	Regular	0	+	tr	0	+	+	±	Strongly positive,	4+
	B. and O'S.		+	+	tr	+	+	tr	Negative,	—
5	Regular	1:25	tr	0	0	+	+	±	Strongly positive, 8+ × 25 = 200+	
	B. and O'S.		+	0	0	+	+	±	Strongly positive	
6	Regular	0	+	0	0	+	+	±	Strongly positive,	5+
	B. and O'S.		+	+	±?	+	+	±	Faintly positive,	±
7	Regular	1:2	+	0	0	+	+	±	Strongly positive, 5+ × 2 = 10+	
	B. and O'S.		+	±	tr	+	±	tr	Negative,	—
8	Regular	1:25	+	±	0	+	+	±	Strongly positive, 3+ × 25 = 75+	
	B. and O'S.		+	+	tr	+	+	±	Weakly positive	
9	Regular	1:100	+	0	0	+	+	±	Strongly positive, 5+ × 100 = 500+	
	B. and O'S.		0	0	0	+	+	±	Strongly positive	
10	Regular	0	+	+	0	+	+	±	Moderately positive,	2+
	B. and O'S.		+	+	±	+	+	±	Negative,	—

* Explanation: 0 = no hemolysis; tr = hemolysis less than 50%; ± = hemolysis between 50 and 100%; + = complete hemolysis.

Table 2 shows the results obtained. Serum 1 gave 8 + by the regular method and a negative result by the Bartlett and O'Shansky modification. Serum 2 gave 125 + by the regular method and was strongly positive by the modification. Serum 3 gave 5 + by the regular method and a negative result by the new modification. Serum 4 gave 4 + by the regular method and a negative result by the modification proposed by Bartlett and O'Shansky. Serum 5 gave 200 + by the regular method and a strongly positive result by the modified method. Serum 6 gave 5 + by the regular method and a faintly positive result by the new modification. Serum 7 gave 10 + by the regular method and a negative result by the modification. Serum 8 gave 75 + by the regular method and 1 + by the modified method. With Serum 9 the regular method gave 500 + and the new modification gave a strongly positive result while Serum 10 gave 2 + and a negative result.

CONCLUSIONS

The Wassermann reaction as regularly done at the Bismarck Hospital may be considered perfectly safe because false positive results have never been observed.

As compared with our regular method the Bartlett and O'Shansky modification gives by far too many negative results.

THE DIFFERENTIATION AND DISTRIBUTION OF THE PARATYPHOID-ENTERITIDIS GROUP

V. OCCURRENCE IN THE HUMAN INTESTINE

Edwin O. Jordan and Ernest E. Irons

From the Department of Hygiene and Bacteriology, University of Chicago

Many investigators, particularly in Germany, have reported finding bacilli belonging to the paratyphoid-enteritidis group widely distributed in nature: in water, ice, milk, chopped meat, sausage, in the healthy organs of normal food animals, in the intestinal contents of various healthy domestic animals and of man, in the dejecta of typhoid carriers and convalescents and of persons ill with other diseases.[1]

Other investigators have not been able to confirm these statements of the "ubiquity" of paratyphoid organisms.[2]

Poppe[3] has given a summary and full bibliography of the observations on the occurrence of the paratyphoid group in foodstuffs and in the organs and intestinal contents of domestic animals, and concludes that the assumption of a widespread distribution is hardly justified by the facts.

There are, of course, many instances on record in which paratyphoid bacilli have been found in the stools of patients supposed to be suffering from typhoid fever, but in most instances of this sort the diagnosis of typhoid has been pronounced either on clinical grounds or on the results of an agglutinative test. Neither method gives assurance that the typhoid bacillus was really the infecting agent; as is well known the clinical symptoms of paratyphoid infection are sometimes such that they cannot be distinguished from those produced by the typhoid bacillus, and the agglutinative reactions of the patient's blood have likewise been shown to be confusing and not fully differentiative.

Received for publication June 12, 1918.

[1] Conradi: Deutsch. med. Wchnschr., 1904, 30, p. 1165. Nieter: München. med. Wchnschr., 1907, 54, p. 1622. Mühlens, Dahm and Fürst: Centralbl. f. Bakteriol., I, O., 1908-1909, 48, p. 1. Conradi: Klin. Jahrb., 1909, 21, p. 421. Rommeler: Deutsch. med. Wchnschr., 1909, 35, p. 886; Centralbl. f. Bakteriol., 1909, I, O., 50, p. 501. G. Mayer: Centralbl. f. Bakteriol., 1909-1910, I, O., 53, p. 234. Komma: Centralbl. f. Bakteriol., 1910, I, O., 55, p. 1. Hübener: Fleischvergiftungen und Paratyphusinfektionen, 1910, p. 66.

[2] Trautmann: Ztschr. f. Hyg. u. Infectionskrankh., 1906, 54, p. 104. Mayer, O.: Klin. Jahrb., 1909, 21, p. 325. Bainbridge and O'Brien: Brit. Med. Jour., 1910, II, p. 1503. Aumann: Centralbl. f. Bakteriol., 1910-11, I, O., 57, p. 310. Zweifel: Centralbl. f. Bakteriol., 1911, I, O., 58, p. 115. Horn and Huber: Ztschr. f. Inf. d. Haustiere, 1911, 10, p. 443. Clausnizer: Arch. f. Hyg., 1913, 80, p. 1.

[3] Arch. f. Hyg., 1913, 80, p. 216.

Eliminating such instances, as well as those of healthy carriers known to have had paratyphoid fever or to have been in contact with cases, there remain a number of instances in which paratyphoid bacilli have been reported as found in the stools of patients who have had genuine typhoid fever.[4]

A certain proportion of such cases are doubtless to be regarded as mixed infections and do not come properly within the scope of this inquiry. The questions that seem to need consideration are (1) how far the frequency of occurrence of paratyphoid bacilli in typhoid stools indicates that secondary infection with these organisms is common, and (2) whether paratyphoid bacilli are as frequently present in normal and diseased human intestines as maintained by some German observers.

One of us has previously put on record a series of observations on the intestinal contents of healthy swine killed in the Chicago stockyards.[5] The results (291 animals) were negative; not a single organism was found that could be identified as belonging to the true B. suipestifer type. The present paper deals with examinations of the feces of typhoid patients and convalescents and of patients suffering from other diseases.

Forty carefully selected cases of active typhoid were examined, mainly in the 2nd to the 4th week of the disease, altho in several instances examinations were made up to the 8th week and later. The total number of feces examinations was 77, the number for an individual ranging from 1-6 depending on the stage of the disease and the character of the findings. B. typhosus was isolated from the blood of 11 of these cases and from the stools of 7 (both blood and stools, 3); in 9 other cases the agglutination was positive; in the remainder (17) no blood examination was made and no agglutination test recorded on the hospital records. Thirteen of the cases occurred during a single water-borne epidemic in a small city when hospital and laboratory facilities were for the time overtaxed. There is no reasonable doubt that all the 40 cases were genuine typhoid.

The Endo medium was used throughout the series of fecal examinations; in many of the cases brilliant green medium, which is especially favorable for paratyphoid bacillus isolation, was used to supplement the former. China green was also used in a few cases. The applicability of the methods used was shown by the ease with which paratyphoid bacilli could be isolated from the stools of a case of paratyphoid B infection previously described by us,[6] and also from a case of paratyphoid A infection clinically resembling mild typhoid and met with by us in the course of our study of active typhoid cases.

[4] Gaehtgens: Arb. a. d. k. Gsndhtsamte, 1907, 25, p. 203. Marmann: Hyg. Rundsch., 1908, 18, p. 1013. Rimpau: Deutsch. med. Wchnschr., 1908, 34, p. 1045. Hübener: Centralbl. f. Bakteriol., 1909, I, Ref., 44, Supp. p. 136.

[5] Jordan: Jour. Infect. Dis., 1918, 22, p. 252.

[6] Jordan and Irons: Jour. Infect. Dis., 1915, 17, p. 234.

In all 649 colonies were picked. Thirty strains from 13 cases were characterized provisionally as paratyphoid-like, that is, dextrose +, lactose —, gelatin not liquefied. These were then subjected to further tests according to the system adopted in an earlier paper.[5]

The following examinations have been made of the stools of typhoid convalescents: 38 men — students in the University of Chicago — who had a definite history of typhoid as follows: in twelve the attack was 12-19 years before the examination; in seventeen, 4-11 years; in eight, less than 3 years and in one, unknown. A single examination by the Endo plate method was made in each case. In all 326 colonies were picked. B. typhosus was not found in any case, nor were any bacilli of the paratyphoid group present. In nearly all instances B. coli and streptococci were the only organisms found. No paratyphoid-like organisms were found. The flora in these healthy convalescents was much less varied than that observed in active cases of typhoid.

Forty-nine women — University of Chicago students — with a definite history of typhoid as follows: in fifteen the attack was 12-25 years before the examination; in twenty-seven, 4-11; in four, less than 3 years and in three, unknown. Four hundred and eight colonies were picked by the Endo plate method, a single examination being made in each case. Bacillus typhosus was not found and the flora was composed mainly of B. coli and streptococci as in the 38 cases just described. Bacilli with paratyphoid-like characters were found in 4 cases. In one of these (Case 156) a second examination after an interval of 6 weeks showed the same organism present. The typhoid attack in this individual had occurred 7 years previously. Several strains of this organism proved identical. On subjecting this bacillus to further tests, it was found to differ in several cultural reactions from the true paratyphoid group, and it did not agglutinate with paratyphoid or enteritidis sera in high dilutions. In the 3 other cases also (Cases 104, 120, 140) in which paratyphoid-like organisms were found the bacilli in no instance agreed in all their cultural characters with true paratyphoid B bacilli and did not agglutinate with B. paratyphosus B and B. enteritidis serums.

The stools in 11 cases of disease other than typhoid were examined. These comprised 3 cases of enteritis, 1 case of carcinoma, 1 of pleurisy, 1 of lobar pneumonia, 2 of ulcerative colitis, 1 of dysentery (neither dysentery nor typhoid bacilli were found) and 2 of an intestinal affection of obscure nature. The same method of plating was used as just described and 156 colonies were picked from the plates made from stools of these patients; on further examination but 2 of these were paratyphoid-like (Cases 52 and 71). One of these strains produced indol and failed to ferment rhamnose or sorbit. The other produced indol and fermented salicin. The intestinal flora disclosed by the mediums used did not differ appreciably from that observed in the typhoid cases: B. pyocyaneus was very abundant in 1 stool and B. fecalis in another; 3 had large numbers of streptococci.

The characters of the paratyphoid-like cultures isolated in these 3 series are shown in Table 1.

SUMMARY AND DISCUSSION

In this series of cases nothing has been found that supports the assumption of a common or widespread distribution of paratyphoid bacilli apart from their association with specific disease conditions. It is true that bacilli with some of the more salient characteristics of

TABLE 1

CULTURAL CHARACTERS OF PARATYPHOID-LIKE BACILLI FROM HUMAN INTESTINES

Case No.	No. of Strains	Paratyphosus B Typical Reaction in Milk	Indol	Salicin	Sorbit	Rhamnose	Remarks
Active Typhoid Cases							
4	1	—	—	+	—	+	Culturally very close to paratyphosus B (but indol +). No trace of agglutination with 1:100 paratyphosus B and enteritidis serums (5,000 titer)
5x	2	—	+	—	+	+	
5y	5	+	+	—	+	+	
24	7	+	+	—	—	—	
32x	5	—	+	—	—	+	
32y	2	+	—	—	—	+	
34x	7	—	+	—	—	+	
34y	3	—	—	—	—	—	
37	2	—	—	—	+	+	
41	1	+	+	+	+	+	
43	5	—	+	+			
45x	1	+	+	—	—	—	
45y	1	—	+	—	—	—	
Old Typhoid History							
104♀	4	+	—	—	—	—	
120♀	1	—	—	+	—	+	Typhoid 7 years before
140♀	2	—	—	+	—	+	Typhoid 4 years before
156♀	2	+	+	+	+	+	Typhoid 7 years before. Two examinations at interval of 6 weeks yielded this organism together with B. coli
Nontyphoidal Diseases							
52	1	+	+	—	—	—	Ulcerative colitis
71	2	+	+	+	+	+	Enteritis

In Table 2 the results are summarized.

TABLE 2

RESULTS OF BACTERIAL FECES EXAMINATION

Fecal Examinations in	No. of Cases	No. of Colonies Picked	No. Paratyphoid-like	No. True Paratyphoid
Active typhoid	40	675	42 (13 strains from 9 cases)	0
Healthy persons (old typhoid history)	87	734	9 (4 cases)	0
Nontyphoidal diseases	11	156	3 (2 cases)	0

paratyphoid bacilli have been found in normal and diseased human intestines, but these have invariably shown cultural deviation from the true paratyphoid type. Moreover, even those that resemble most closely paratyphoid bacilli in their cultural characters have failed to agglutinate with highly potent specific serums prepared from typical members of this group. No true paratyphoid bacilli have been found.

As seen in Table 1, paratyphoid-like organisms are isolated more frequently from the stools of active typhoid cases than from those of healthy persons who had had typhoid some years previously. The intestinal flora of the latter in fact does not differ appreciably from that of normal individuals not known to have had typhoid. It is worth noting that the 4 persons with old typhoid history in whose stools paratyphoid-like bacilli were found were all women (4 of 49 examined), while none of the men of the same series (38) harbored such organisms. The larger proportion of women than of men typhoid carriers is well known.

The 19 strains of bacilli denominated as paratyphoid-like (dextrose +, lactose—, gelatin not liquefied in this series have given paratyphoid-like reactions in other mediums as follows:

Milk	9
Indol	7
Salicin	11
Sorbit	6
Rhamnose	11

Combining these with similar tests made on 18 strains of paratyphoid-like organisms isolated from healthy swine intestines,[5] it appears as if the milk and indol reactions were better fitted for differentiating these organisms from the true paratyphoid bacilli than the fermentation results in salicin, sorbit and rhamnose. More than half of these bacilli (22 of 37) fail to ferment salicin, in this respect agreeing with true paratyphoid organisms, but only 14 of 37 give the paratyphoid reaction in milk and only 16 of 37 fail to produce indol.

It will be noticed (Table 1) that in a number of active typhoid cases different varieties of paratyphoid-like bacilli were found in the stools of one and the same individual (Cases 5, 32, 34, 45). This corresponds with the generally heterogeneous bacteriologic characters of such stools. It is evident that the bacterial flora of typhoid stools is more varied than that of normal stools. There is, however, no reason for assuming without further investigation that this condition is peculiar to the intestinal tract of typhoid patients. The few observations here recorded on the stools of persons suffering from other disorders, especially disorders of the alimentary tract, indicate that the proportion of bacterial abnormalities in such cases is probably not very different from that in typhoid cases.

The occurrence in typhoid stools of such paratyphoid-like bacilli, or, as some writers call them, paracolon bacilli, has been noted by

several investigators.* Seiffert[7] has described 3 such strains. Fletcher[8] found in the stools of soldiers convalescent from diseases of the dysentery and enteric groups a number of organisms that he called "anomalous organisms of the Salmonella group." These were generally to be distinguished by their lack of motility or by their power to form indol. Extensive fermentation tests were not made. Agglutination reactions with paratyphoid sera were negative. Fletcher found such organisms at times "very common."

Agglutination tests have been made repeatedly with all the strains here mentioned. Rabbit serums have been used prepared with several strains of B. paratyphosus B, with 2 strains of B. paratyphosus A, 2 of B. suipestifer and 2 of B. enteritidis. These serums have agglutinated the homologous organisms in about 1:4,000 to 1:5,000 dilutions (in the case of B. enteritidis 1:20,000). In no instance have any of these culturally anomalous intestinal strains agglutinated in dilutions higher than 1:100. It has been exceptional indeed to observe even a trace of agglutination in a 1:100 dilution.

A serum prepared with one of these intestinal cultures, 32x (titer 1:1,000) did not agglutinate other intestinal strains and did not agglutinate 2 other strains isolated at the same time from the same cases and showing similar cultural characteristics. Serums were prepared with 2 other strains from other cases with precisely similar results. The titer reached with these 3 strains was not very high (1:1,000 to 1:2,000), but in no single instance was agglutination observed in 1:100 dilutions with any of the other intestinal strains here enumerated. This was true in the case of those strains showing close cultural resemblance as well as those showing considerable divergence. In this respect the behavior of these "paratyphoid-like" organisms is not very different from that of colon bacilli.

* The term paracolon bacillus might well be restricted to such organisms as ferment lactose with or without gas production, but fail to give certain typical coli-like reactions such as indol production, altho in other respects closely related to the true B. coli. The term paratyphoid-like is awkward, but has some advantages when used to designate the nonlactose fermenters.

[7] Ztschr. f. Hyg. u. Infectionskrankh., 1909, 63, p. 273.

[8] Jour. Roy. Army Med. Corps, 1918, 30, p. 57.

FAILURE TO OBTAIN POTENT SERUM FROM HORSE INJECTED WITH POLIOMYELITIC MATERIAL

SAMUEL G. DIXON AND JAMES B. RUCKER, JR.

From the Laboratories of the Pennsylvania Department of Health, Philadelphia

When so much is being done on the subject of immunity in epidemic poliomyelitis and so much more has yet to be learned concerning it, it may be well to record the negative results of an experiment in order that the same line of work may not be gone over again when it has proven by painstaking experiment to be of no value.

Early in 1917 it was determined to attempt immunization of a horse by injecting the filtrate of known poliomyelitic material under the skin, over a long period of time. Accordingly, injections were begun on March 10, 1917, with the unheated filtrate of a 1:50 emulsion of poliomyelitic brain and cord. This poliomyelitic material had been kept in 25% glycerol for about five months.

The horse received gradually increasing doses of this filtrate, beginning with 5 c c and working up to 120 c c at the end of a month, when the first bleeding was made. With the serum, Monkey 164 was injected in 5 c c doses as follows:

May 2: Under the skin morning and night; then again on May 3 and 4; 1 dose May 5, and 1 May 7; 2 doses on the 4 following days and 1 dose May 12.

May 7: Injected into right side of brain 1 c c emulsion of poliomyelitic cord from Monkey 155.

May 5-11: Moves slowly; unsteady.

May 12: Both legs flaccid; other members unaffected; nervous irritability.

May 13: Completely paralyzed in all members. Killed.

It was thus shown that the serum of the horse injected for six weeks with the unheated filtrate of poliomyelitic central nervous system was of no avail as a preventive against experimental poliomyelitis. The horse in the meantime had been receiving every few days steadily increasing doses of the poliomyelitic material, and after five months was bled again and the serum used in another attempt at immunization of monkeys against poliomyelitis.

MONKEY 166.—Injected subcutaneously with 5 c c morning and night on July 31.

Aug. 2: Not well, serum not given.

Aug. 3 and 4: Well, 10 c c under skin.

Received for publication June 20, 1918.

Aug. 6: Injected intracerebrally with 1 c c of virus Monkey 164.

Aug. 7-10: 10 c c serum under skin daily. Well.

Aug. 11: Paralyzed in left arm, loss of power in right leg. Injected 3 c c serum into spinal canal after withdrawing 1.5 c c spinal fluid. This was followed by pressure symptoms and weakness in legs, so that animal could not sit up or walk; staring eyes; recovered equilibrium in about 10 minutes.

Aug. 12: Complete paralysis in all members. No treatment.

Aug. 13: Died last night.

Monkey 167.—July 31. Injected subcutaneously 5 c c, morning and night, serum of horse immunized with unheated filtrate poliomyelitic material.

Aug. 2: Not well, no serum treatment.

Aug. 3 and 4: Well, 10 c c under skin daily.

Aug. 6: Injected intracerebrally with 1 c c of virus Monkey 164.

Aug. 7, 8, 9: 10 c c serum under skin daily. Well.

Aug. 10: Hair ruffled; 8 c c serum injected into left femoral vein under ether.

Aug. 11: Animal shaky, with nervous twitching of head and limbs; 3 c c of serum injected into spinal canal, 2 minutes after which pressure symptoms occurred as manifested by rapid breathing, staring, shifting eyes, extreme weakness of legs and arms. Recovered in 5 minutes.

Aug. 12: Complete paralysis in all members. No treatment.

Aug. 13: Killed.

Monkey 168.—July 31. Received subcutaneously 5 c c, morning and evening, serum of horse immunized with filtrate of poliomyelitic material.

Aug. 3 and 4: 10 c c under skin in 2 places on each day.

Aug. 6: 1 c c virus Monkey 164 intracerebrally.

Aug. 7, 8, 9: 10 c c horse serum subcutaneously each day.

Aug. 8: 1 c c spinal fluid withdrawn and 2 c c horse serum injected.

Aug. 11: Animal seems slightly slow in movement, otherwise well, 2.5 c c horse serum into spinal canal. No pressure symptoms.

Aug. 12: Animal somewhat weak but no paralysis. Injected 3 c c horse serum into spinal canal. Pressure symptoms occurred but disappeared in about half an hour and monkey was able to climb cage.

Aug. 13: Animal completely paralyzed. Died at noon.

The experiments with 3 monkeys show that antibodies of sufficient potency to protect against experimental poliomyelitis had not developed in the serum of a horse injected over a period of five months with poliomyelitic material.

Finally a trial was made of the serum of the horse after seven months injection with poliomyelitic material. Two weeks prior to intracerebral inoculation with the virus, 10 c c serum were given subcutaneously daily for a week, then 5 c c subcutaneously and 2 c c intraspinally each day for a week.

This treatment was carried out in every detail, but notwithstanding this, the result in Monkey 176 proved that the horse serum contained no immunizing bodies after it had received injections of the filtrate of unheated poliomyelitic material for seven months.

MONKEY 176.—The animal was injected under the skin with 10 c c horse serum Nov. 26; Nov. 27, 28, 30, Dec. 1 and 3 with 5 c c twice daily.

Dec. 4: Began intraspinal injection of horse serum 2 c c, giving also 5 c c under the skin. This was continued on 5 successive days. On Dec. 10 the animal was injected intracerebrally with 0.5 c c emulsion of cord of Monkey 172. At same time 2 c c of horse serum was injected into spinal canal.

Dec. 12, 13, 14: 2 c c horse serum into spinal canal, 5 c c under skin each day

Dec. 15: Irritable since yesterday; flaccid paralysis of legs. No treatment.

Dec. 17: Moribund; killed.

The serum of the horse obtained after 7 months of injections was used also as a curative agent after infection, but before the first symptoms of poliomyelitis such as ruffling of the hair and nervous irritability manifested themselves. Five animals and a control were used in this experiment.

MONKEY 170.—This animal received into the brain 0.5 c c emulsion of cord and pons of Monkey 168 and 2 c c of horse serum intraspinally on Nov. 19.

Nov. 20: 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 21: 2 c c horse serum into spinal canal, 5 c c into femoral vein.

Nov. 22: 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 23: 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 24: 2 c c into spinal canal, 5 c c under skin.

Nov. 25: Paralyzed in arms, legs, back and neck muscles.

Nov. 26: Moribund; killed.

MONKEY 171.—Received the same treatment as Monkey 170 on Nov. 19.

Nov. 20: 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 21: 2 c c horse serum into spinal canal, 5 c c into femoral vein.

Nov. 23: 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 24: A little shaky, otherwise active and well; 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 25: Down completely; muscles of arms, legs, back and neck show flaccid paralysis.

Nov. 26: Dead.

MONKEY 172.—Control. Received 0.5 c c emulsion of cord and pons of Monkey 168, Nov. 19.

Nov. 23: Slow in movement, rather shaky.

Nov. 24: Paralyzed completely in legs; back muscles weak; arms not affected.

Nov. 25: Paralyzed in arms, back, neck and legs, moribund.

Nov. 26: Dead.

MONKEY 173.—Received same treatment as 172 on Nov. 19.

Nov. 22: Received 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 23: 2 c c horse serum into spinal canal, 5 c c into femoral vein, also on Nov. 24.

Nov. 25: Down on all fours. Paralyzed.

Nov. 26: Dead.

MONKEY 174.—Received same treatment as Monkey 172 on Nov. 19.

Nov. 22: 2 c c horse serum into spinal canal, 5 c c subcutaneously.

Nov. 23: 2 c c horse serum into spinal canal, 5 c c into vein.

Nov. 24: Very shaky, 2 c c horse serum into spinal canal, 5 c c subcutaneously.

Nov. 25: Paralyzed in all members; neck and back paralyzed.

Nov. 26: Dead.

Monkey 175.—Received same treatment as Monkey 172, Nov. 19.

Nov. 22: 2 c c horse serum into spinal canal, 5 c c under skin.

Nov. 23: 2 c c horse serum into spinal canal, 5 c c serum into vein.

Nov. 24: Lies on bottom of cage, almost moribund. Killed.

The experiment with six monkeys shows the serum to have no curative value.

Microscopic examination of sections of the cords of all the monkeys that died showed typical lesions of epidemic poliomyelitis.

Neutralization experiments on monkeys with 0.5 c c of salt solution emulsion of the virus, 1 to 10, incubated at 38 C. for one-half hour with 1 c c of the serum of the 7 months' immunized horse, showed the serum to have no neutralizing value, the treated monkeys becoming paralyzed as soon as the control.

The reactive response of the horse to the injections of virus, thus seemingly evidencing the activity of the latter as an infective agent, was shown by a rise in temperature of from 1-5 degrees, after each injection; general muscular tremors, after many of the injections; and in one instance, after the injection of 120 c c of filtrate, the animal fell down, pulse became rapid, respiration labored, mucous membranes congested, and was very nervous and hypersensitive to sudden noises of any kind. These general reactions were probably anaphylactic in nature, and may not have been due at all to the specific effect of the poliomyelitis virus as such, but the exact nature cannot be known.

The horse began treatment by receiving 1 c c of the filtrate, and at the end of the 7th month when the last test of the potency of its serum was made on monkeys, was receiving 500 c c of the filtrate subcutaneously.

STUDIES IN THE METABOLISM OF PATHOGENIC ACTINOMYCETES (STREPTOTHRICES). I

SELMAN A. WAKSMAN

From the Research Department of the Cutter Biological Laboratories, Berkeley, Calif.

INTRODUCTION

A great deal of confusion exists on the subject of classification as well as on the biochemical activities of the actinomycetes. Different organisms have been often studied under the same name, and the same organism has been studied under different names. Not only the species have been mixed up, but investigators could not agree even on the generic name, and the names Actinomyces, Streptothrix, and Nocardia have been used indiscriminately for designating often a single species. This is due largely to the fact that morphological studies of these organisms were made almost exclusively, and since morphologically and even culturally many species largely resemble one another, the reason for confusion becomes evident. Not only the older investigators, but even the more modern, which have at their possession numerous biochemical methods that can be applied advantageously to the study of these organisms, are making errors in their attempt to classify them. For example, in the "Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types" for 1917,[1] the actinomycetes are divided into two genera: Actinomyces, usually parasitic organisms; no aerial hyphae or conidia; Actinomyces bovis taken as type; and Nocardia, producing aerial mycelium. This classification will not hold, since many pathogenic organisms, when grown on inorganic mediums, will produce aerial hyphae and conidia, and even the type taken, namely, Actinomyces bovis, will produce a scant aerial mycelium on inorganic medium. As to the pathogenicity, there is no doubt that many pathogenic actinomycetes are isolated from the soil and are true saprophytes. The presence or absence of aerial mycelium, as attempted by the committee for generic differentiation, size and shape of spores, as attempted by Krainsky[2] and Conn,[3] for specific differen-

Received for publication, June 17, 1918.

[1] Winslow, Broadhurst, Buchanan, Rogers, and Smith: Jour. Bacteriol., 1917, 2, p. 505.
[2] Centralbl. Bakteriol., II, 1915, 41, p. 649.
[3] N. Y. Agr. Exp. Station, Bull. 60.

tiation; or, pigment production, as attempted by some of the older investigators, cannot be used as primary factors in dividing these organisms; these characters should be used only as secondary points in the differentiation of the different species. Biochemical studies of these organisms will no doubt throw a great deal of light on the nature of the organisms and thus help to bring about a more thorough classification.

The term "Actinomyces" will be applied in this paper to the pathogenic forms, both for animals and plants, as well as to saprophytic forms, from many of which the pathogenic can hardly be differentiated morphologically, culturally and chemically. A complete bibliography on the subject of the nature and activities of the actinomycetes is found in the recent papers of Krainsky,[2] Waksman and Curtis,[4] and Conn.[3] All these investigators have used, in addition to organic mediums, also inorganic mediums for the growth of the actinomycetes, and thus made an important step forward in the study and classification of this very interesting and large group of organisms. While Krainsky and Conn used largely the morphologic characters in classifying these organisms, Waksman and Curtis attempted to apply also biochemical studies. None of them have succeeded so far in offering a good basis for classifying the actinomycetes, but their work is a step forward in the direction of the proper study of the organisms. A detailed report of the biochemical activities of the actinomycetes isolated from the soil will soon appear (Waksman[5]). This paper, therefore, deals only with the pathogenic forms, plant and animal.

ORGANISMS STUDIED

Actinomyces bovis Harz (Syn. Streptothrix bovis).
Actinomyces asteroides (Eppinger) Gasperini (Syn. Streptothrix Eppingeri Rossi-Doria).
Actinomyces madurae (Vincent) Lehmann and Newmann (Syn. Streptothrix madurae Vincent).
Actinomyces hominis Boström.

These four cultures were obtained from Dr. K. F. Meyer of the Hooper Foundation, University of California, who received them originally from the Pasteur Institute in Paris and the New York Museum of Natural History.

Actinomyces scabies (Syn. Oospora scabies Thaxter), isolated from several sources and received from different investigators (the potato-scab strain was used in this work).

Actinomyces psolensis Taubenhaus,[6] pathogenic to sweet potatoes and isolated also by the author from the soil. The culture of Dr. Taubenhaus was used in this work.

[4] Soil Science, 1916, 1, p. 101.
[5] Soil Science (to be published soon).
[6] Jour. Agr. Research, 1918, 13, p. 437.

METHODS

Only the analytic data are given in this paper. Although the organisms were grown successfully on a number of organic mediums only those found useful for the growth of these organisms are mentioned.

For the study of hemolysis, blood agar was used; 10% of sterile defibrinated rabbit blood were added to sterile 2.5% nutrient (sugar free) agar having a reaction of + 1.0; the agar was well mixed with the blood, then distributed into sterile tubes, which were slanted, allowed to cool, then incubated to insure sterility; 15% of Gold Label gelatin in distilled water and adjusted to neutrality was used for the study of gelatin liquefaction. ($P_H = 7.0$ or hydrogen ion concentration $= 10^{-7}$; the colorimetric method was used.) The gelatin was poured into sterile Petri dishes, these were cooled, inoculated and allowed to stay at room temperature (18-20 C.). Skimmed milk sterilized at 15 lbs. for 30 minutes was used for the study of coagulation of the milk and proteolytic action on the milk proteins. For serum liquefaction, Löffler's blood serum mixture was used. For the study of the proteolytic enzymes of the organisms, 2 c c of the culture were added to 10 c c of the 15% gelatin in distilled water or to 10 c c of sterile skimmed milk; 1 c c of toluol was added to each tube, to insure sterility. All the cultures, except the gelatin plates, were incubated at 37 C.

The proteolytic action of the actinomyces and their enzymes was followed by the determination of the amino-nitrogen of the culture, by the use of the Van Slyke apparatus.

BLOOD AGAR CULTURES

A. madurae: A good dark-brown growth was obtained in 48 hours. A sharply defined, transparent, completely hemolized zone from 2-4 mm. in width around the growth in 15 days.

A. hominis: Good growth in 24 hours. Distinct hemolysis in 4 days, 3-4 mm. clear zone in 15 days.

A. bovis: Good spreading growth, faint hemolysis; clear zone in 15 days only 1-2 mm.

A. asteroides: A thin brownish smear appears in 24-48 hours, no hemolysis in 6 weeks. Gray aerial mycelium appears in 8-10 days.

A. psolensis: Greenish fine network on surface of agar, no hemolysis in 6 weeks.

A. scabies: No growth on blood agar.

Blood agar was found to be a very good medium for actinomyces, particularly for the animal pathogens. The hemolysis is characteristic of the species, and, as will be seen later, is a property of many actinomycetes which have a strong proteolytic power. The presence or absence of hemolysis can no doubt serve as a distinguishing feature in the identification of the organisms. Aerial mycelium was produced only in the case of the A. asteroides.

BLOOD SERUM CULTURES

A. madurae: Growth in 48-72 hours; serum dissolved in 6 days around the growth.

A. hominis: Good growth in 48-72 hours; serum dissolved in 6 days around the growth.

A. bovis: Minute yellowish colonies, serum not dissolved in 6 weeks, only becoming transparent.

A. asteroides: Very good growth in 48-72 hours. Serum not dissolved in 6 weeks, has not even become transparent.

A. psolensis: Good growth. serum not dissolved.

A. scabies: Scant growth, serum not dissolved.

Löffler's blood serum mixture forms a good medium for the cultivation of many actinomycetes, particularly the forms pathogenic for animals; the liquefaction of the serum can be taken as a characteristic point in differentiating the organisms. No aerial mycelium was produced on any of the organisms, when grown on this medium. By comparing the results of the growth of the organisms, one can readily observe that the hemolysis of the blood and liquefaction of the serum run parallel and are characteristic properties of the same organisms.

Gelatin (15% in Distilled Water) Cultures

A. madurae: Gelatin begins to liquefy rapidly (6 days) around the colony, and in 15 days (18-20 C.) the liquefied zone is 5-12 mm. wide. No pigment produced.

A. hominis: Gelatin begins to liquefy in 3-4 days, and in 15 days liquefied zone is 4-7 mm. wide. No pigment produced.

A. bovis: Gelatin does not liquefy till very late. In 15 days, there is a faint liquefied zone (¼ mm.) around the growth. No pigment produced.

A. asteroides: No liquefaction of the gelatin, even in 30 days. No pigment production. Growth present (gray, thin).

A. psolensis: Gelatin slowly liquefied; in 15 days, clear zone is 4-5 mm. wide. No pigment produced.

A. scabies: Gelatin is not liquefied in 30 days; growth very slow.

The liquefaction of gelatin, which would designate the production of a proteolytic enzyme, runs parallel with the action of the organisms on blood agar and blood serum. No aerial mycelium was produced in any of the cultures studied here; no pigmentation took place in any of the cultures reported above, although, as can be seen in another place (Waksman and Curtis[4]) this gelatin is pigmented by a number of actinomyces. Lehmann and Neumann[7] report a limited liquefaction of gelatin by A. bovis; A. madurae is reported by Vincent as not liquefying gelatin (Lehmann and Newmann); while Petruschky[8] reports that it does liquefy gelatin; A. asteroides does not liquefy gelatin, after Petruscky.

[7] Atlas and Principles of Bacteriology, Pt. II, 1901, p. 438.

[8] Handbuch der pathogenen Mikroorganismen, Kolle and Wassermann, 1913, 5, p. 267.

MILK CULTURES *

The data obtained from the growth of actinomyces on milk at 37 C. are presented in Table 1.

TABLE 1

THE GROWTH OF PATHOGENIC ACTINOMYCETES ON MILK
Total Nitrogen in 10 c c of Milk = 59.6 mg.

Name of Organism	Clot Days	Peptonization		$NH_2 - N$ in 10 c c 15 days' incubation	Per Cent. of Total Nitrogen
		Begins, Days	Complete, Days		
Control	—	—	—	1.33	2.2
A. madurae	3	4	15	35.63	59.8
A. hominis	6	8	15 Half peptonized	19.95	33.5
A. scabies	9	10	—	(Not determined)	—
A. bovis	—	Digestion without previous coagulation		9.98	16.8
A. poolensis	–	Digestion without previous coagulation		12.69	21.3
A. asteroides	—	–	—	5.72	9.6

Milk can be very readily used for the differentiation of the pathogenic actinomyces. The ability to coagulate and then peptonize the milk differs with the different organisms. No doubt that the acclimatization of the organisms plays an important part in this respect, as will be brought out later.[5] The most important point to be observed in this experiment is the proteolytic action of the different actinomycetes. A. madurae producing most hemolysis, being the strongest gelatin liquefier, digesting more blood serum than the others, produces also the quickest coagulation of the milk and strongest digestion of the milk proteins: nearly 60% of the proteins of the milk have been digested and converted into amino-acids and other simple nitrogenous compounds showing a large content of amino-nitrogen. A. hominis, which was found, in the previous experiments, a somewhat weaker proteolytic organism than A. madurae, clotted the milk only in 6 days and digested the milk proteins almost to a half the extent than the latter organism. A. bovis, which was found to possess still weaker proteolytic activities than the first two organisms, did not clot the milk at all, but digested it with the production of amino-nitrogen amounting to 16.8% of the total nitrogen of the milk. A. asteroides, which was found to have almost no proteolytic activities, exerted no visible action whatsoever on the milk in 15 days, at 37 C., although it made a good growth on it. When the amino-nitrogen of the milk was determined it

* On repeated inoculation some of the organisms were found to behave in a slightly different way upon milk. This will be brought out later[5] and explanations suggested.

was found to increase from 2.2-9.6% of the total nitrogen, showing that some proteolysis has taken place, although no visible change was observed. A. psolensis behaved in a manner similar to A. bovis, but producing a somewhat stronger proteolytic action. A. scabies clotted the milk in 9 days, but produced very little decomposition of the clot in 15 days.

Lehmann and Neumann stated that A. bovis does not change milk in 8 days; the milk culture used in the above experiment showed hardly any change in 8 days, but in 15 days the culture became clear, almost transparent (faint turbidity). Petruschky reported that A. madurae coagulates milk, which is later redissolved, and A. asteroides produces no coagulation of the milk, although a red-brown growth is obtained; these results are confirmed by the writer.

The reaction of the milk was tested, after the 15 day period, to see whether the clotting may not be due to acid production in the milk; with sodium alizarine sulfonate as an indicator, the medium was found to be in all cases alkaline. The question of reaction changes in culture mediums of actinomycetes as well as a more detailed study in the nitrogen and carbohydrate metabolism of these organisms will be published elsewhere.[5]

To study the production of a rennet-like and of proteolytic enzymes by the actinomycetes in milk the following procedure was used:

Two c c of the milk, on which the organisms grew for 15 days (digested or not) was added to tubes containing 10 c c of sterile skimmed milk or 10 c c of sterile gelatin (15% in distilled water, dissolved, adjusted to neutrality, filtered and sterilized for 30 minutes at 100 C. on 3 consecutive days). One c c of toluene was added to each tube to prevent any growth of the organism; the tubes were stopped with rubber stoppers, shaken, and incubated at 37 C.

The results are presented in Tables 2 and 3. As a control, the same amount of inoculum, boiled for 5 minutes, was added to the gelatin and milk, treated as stated above and also incubated at 37 C. The enzyme cultures were incubated for 5 days, then the amino-nitrogen was determined in all of them to see whether any proteolytic action has taken place. The gelatin tubes were placed for 1 hour on ice, and if the tube remained liquid, the liquefaction was taken as positive.

All the 4 strains, pathogenic for animals, seem to produce a proteolytic enzyme, when grown on milk, but the quantity of the enzyme is distinctly different for the different organisms; A. bovis and A. asteroides did not produce any rennet-like enzyme in this experiment, but the culture of the first organism contained a fairly good proteolytic enzyme and the culture of the latter a very weak enzyme or only

a trace of it; A. madurae and A. hominis produced both a rennet-like and a strong proteolytic enzyme; the action of this enzyme was different for the 2 organisms: A. madurae, being the stronger proteolytic organism, produced also a larger amount or a more active enzyme.

TABLE 2

THE ACTION OF THE ENZYMES OF ACTINOMYCETES (OBTAINED FROM 15 DAY MILK CULTURES) ON MILK

Organism	Clot Production in Days		NH_2 - N mg. in 10 c c	
	Control	Enzyme	Control	Enzyme
A. madurae	—	2	7.05	13.79
A. hominis	—	2	4.43	7.76
A. bovis	—	?	2.77	8.58
A. asteroides	—	—	2.06	2.48

Similar observations can be made from Table 3.

TABLE 3

THE ACTION OF THE PROTEOLYTIC ENZYME OF ACTINOMYCETES (OBTAINED FROM 15 DAY MILK CULTURES) ON GELATIN

Organism	Gelatin Liquefaction in Days		NH_2 - N mg. in 10 c c	
	Control	Enzyme	Control	Enzyme
A. madurae	—	2	10.88	15.39
A hominis	—	2	8.27	13.11
A. bovis	—	3	6.61	10.83
A. asteroides	—	— (10 days not liquefied)	5.89	5.95

The action of the proteolytic enzymes obtained from the milk is alike on gelatin and on the milk proteins, showing that the enzyme is probably the same, particularly since the reaction of the milk and of the gelatin were the same (about neutral).

The organisms were also grown on several synthetic mediums.

A detailed discussion of these will be found elsewhere. Mention will only be made of those mediums that can be used for the growth of some pathogenic actinomycetes. Saccharose, dextrose, and glycerol synthetic agars and Krainsky's glucose agar are reported here. As a basis for the first 3 mediums the Czapek's solution agar (without the saccharose) was used. The composition of this medium is as follows:

Distilled water	1,000	c c
$NaNO_3$	2.00	gm.
$MgSO_4$	0.50	gm.
K_2HPO_4	1.00	gm.
KCl	0.50	gm.
$FeSO_4$	0.01	gm.
Agar	15.00	gm.

To this medium 30 gm. of saccharose, dextrose, or glycerol were added, the materials were dissolved by boiling and, without further adjustment of the reaction, filtered through absorbent cotton, tubed, and sterilized at 15 lbs. pressure for 30 minutes.

Krainsky's glucose agar has the following composition:

Distilled water	1,000 c c
Agar	15.0 gm.
Glucose	10.0 gm.
Asparagin	5.0 gm.
K_2HPO_4	0.5 gm.

A. madurae grows equally well (growth spreading and growing deep into the medium, scant white mycelium on saccharose and glycerol) on all the four mediums, particularly where dextrose or glycerol are present as a source of carbohydrates.

A. hominis grows very well on all the four synthetic mediums, producing a heavy white mycelium.

A. bovis grows slowly on all the four mediums, never producing any very extensive growth.

A. asteroides grows well on all the four mediums, best on the dextrose synthetic agar, least on the saccharose synthetic agar (growth particularly heavy on the Czapek's synthetic solution agar, when saccharose is replaced by dextrose).

SUMMARY

Blood agar is a very good medium for the growth of pathogenic actinomycetes, a good growth being obtained in 24-72 hours when incubated at 37 C.

The production of hemolysis of the blood on blood agar, the liquefaction of blood serum, the clotting and subsequent peptonization of the milk, the liquefaction of gelatin, run parallel; the organism that produces most hemolysis, produces liquefaction of the blood serum and gelatin and a greater digestion of the milk proteins; the organism that does not produce any hemolysis of the blood does not liquefy the blood serum and the gelatin, does not clot the milk and has only a small action on the milk proteins. These characters can be used advantageously in the identification and classification of the actinomycetes.

Some pathogenic actinomycetes grow readily on synthetic mediums.

A CASE OF RAT-BITE FEVER

PLATE 5

RUTH TUNNICLIFF AND KATHERINE M. MAYER

From the John McCormick Institute for Infectious Diseases and the Children's Memorial Hospital, Chicago

A streptothrix—Streptothrix muris-ratti—has been isolated from the blood of 3 patients with rat-bite fever (Schottmüller,[1] Blake,[2] and Litterer[3]) and observed in the fresh blood in a fourth case by Tileston.[4] Similar organisms, culturally and morphologically, have been found in bronchopneumonia of white rats by Tunnicliff.[5] Another streptothrix—Streptothrix putorii—has been isolated by Dick and Tunnicliff[6] from the blood of a patient bitten by a weasel. The clinical picture of this case was similar to that of rat-bite fever, but the streptothrix differed from Streptothrix muris-ratti culturally in growing more profusely and in producing a grayish-yellow color on dextrose-blood agar, and in growing in plain and dextrose broth and in milk. It differed morphologically in not forming long and wavy filaments.

The present case of rat-bite fever, which ended in death, occurred in a colored girl, aged 13 days, who entered the Children's Memorial Hospital, March 11, 1918, in the service of Dr. Parmalee. Nine days before, the patient was bitten by a rat on the face. The upper lip and gum were cut through and the left cheek lacerated. On entrance there was edema of the eyelids; the left cheek and upper lip were covered with small, irregular, not depressed, scarlike areas; the right cheek was red, indurated and firm from the nose to the angle of the mouth and external canthus of the eye, and was apparently painful, the upper lip being deformed. The cervical glands were palpable. The physical examination otherwise negative. Leukocyte count, 32,000, temperature, 101 F.

March 15: Pus discharging from the right nostril. Temperature normal.

March 21: Temperature rose to 101 F.

March 22: Wassermann reaction negative. An eruption has appeared on the chest and abdomen of bluish red, sharply defined macules, about 3 cm. in diameter. They were somewhat obscure on account of the color of the skin.

March 23: Blood culture, negative.

Received for publication July 1, 1918.

[1] Dermat. Wchnschr., 1914, 58, Suppl., p. 77.
[2] Jour. Exper. Med., 1916, 23, p. 39.
[3] Jour. Tenn. State Med. Assn., 1917, 10, p. 310.
[4] Jour. Am. Med. Assn., 1916, 66, p. 995.
[5] Jour. Infect. Dis., 1916, 19, p. 767.
[6] Ibid., 1918, 23, p. 183.

March 25: The eruption had faded; hemoglobin, 70%; red blood corpuscles, 4,200,000; leukocytes, 17,000; polymorphonuclear cells, 74%; large lymphocytes, 14%; small lymphocytes, 12%.

March 27: Leukocytes, 18,800.

March 28: Patient died at 4 a. m.

The necropsy 11 hours after death by Dr. R. F. Austin showed general anemia of the viscera; malnutrition; healed bite wounds of left cheek; uric acid infarcts of kidneys.

Section of the tissues were stained by the Gram and also by the Levaditi methods. The heart, spleen and kidney sections showed no changes. The lung showed a little bronchopneumonia, and many small streptococci, large coccobacilli, and a few filaments were present in the lung tissue. The liver showed some hyperemia. There were hemorrhages in the suprarenals. Sections of ganglions near the suprarenals, which were stained by Levaditi's method, showed slight degeneration of the ganglion cells and some cells contained in their nuclei short and long filaments (5-10 mikrons), some being quite wavy; round swellings or attachments were observed on occasional filaments. and one end was at times broader than the other; one filament appeared to branch (Fig. 3). No spirochetes were seen in any of the sections. Great care must be taken in Levaditi sections not to mistake nerve and connective tissue fibers for spirochetes.

Sections were made of ganglions in the region of the suprarenals, stained by Levaditi's method, from 2 adults to determine better whether the filaments seen in the ganglion cells in this case of rat-bite fever are organisms or not. As no such filaments have been observed in the 2 controls and as the filaments correspond closely to those observed in the blood during life and in the cultures, it is possible that they are organisms. If they are organisms their presence in the ganglion cells may have had something to do with the death of the patient, the lesions in the other organs being almost negligible.

Smears from the bone marrow showed streptococci and filaments; stained by Leishman's and Tribondeau's modification of Fontana's silver stain, no spirochetes were found in smears from any of the organs.

Blood smears made March 22 when the eruption was present, but the temperature reduced, showed inside the polymorphonuclear leukocytes, filaments 5-10 mikrons in length. They were fairly straight; one end was often wider than the other; one appeared to branch. About 5 were observed in a blood smear and were best seen in stains with carbol-thionin followed by carbol-gentian-violet (Fig. 1). A few were present outside the cells. They were not seen in smears made the following day. Dark fluid examination of blood drawn March 23 and 27 failed to demonstrate any organisms.

Blood cultures made March 22 were negative; this was 24 hours after the onset of the attack, and at that time the organisms found in smears were largely inside the leukocytes, which may account for the failure of growth.

Cultures from the heart blood at death were made on goat blood-agar plates, ascitic broth and Loeffler's blood serum. After 48 hours' incubation all showed a growth of a streptothrix and large gram-negative coccobacilli, such as seen in the sections of the lung. On the blood-agar plates, which showed about 4-10 colonies of the streptothrix to one of the coccobacillus, the streptothrix grew as minute, dry, grayish colonies, becoming larger and moister on cultivation, while the coccobacillus produced a large grayish moist colony. A streptococcus producing green colonies on blood agar was isolated from the lung.

The streptothrix from the blood of this case is nonmotile, slender, curved and branching (Fig. 2). Branching is not frequent and seen

only in cultures about 18 hours old, when the growth is just visible. The organisms vary from 1-10 mikrons in length. The longest forms are seen in early cultures, about 18 hours, the short forms only being present later; the organisms often appear broader at one end than the other and S-shaped forms are seen; ball-shaped swellings are observed especially when the organism branches. A few long and wavy filaments were observed only in the early cultures in ascitic broth. The organism is gram-negative, stains with ordinary stains and is not acid fast. It grows both aerobically and anaerobically, and growth generally occurs in 24-48 hours on blood agar and Loeffler's blood serum as discrete colorless, grayish-white pinpoint, circular, elevated colonies with sharp margins. Later the growth becomes moist and confluent. It does not produce any pigment. The medium is not changed. Growth in ascitic broth is slight but whitish and flocculent, the medium not remaining clear. There is slight growth only in plain and dextrose broth and milk, and on plain agar, dextrose agar and potato. The streptothrix ferments dextrose, raffinose, maltose and leontose, but not salicin, lactose, inulin and mannite. It is killed by exposure to 65 C. for one half hour.

The growth on one blood-agar slant injected intravenously in a rabbit made it ill but did not kill it or produce any special symptoms. No organism could be demonstrated in the blood.

The coccobacillus ferments no sugars and was not virulent for guinea-pigs. The streptococcus from the lung fermented salicin, dextrose, lactose and saccharose and was nonpathogenic for guinea-pigs.

The patient's blood was studied for opsonins and agglutinins for Streptothrix putorii, but none were demonstrated. Further experiments could not be made because of lack of blood. Agglutinins (1:10) for the homologous streptothrix were present in the blood of the rabbit 1 month after inoculation, but not for Streptothrix putorii.

The streptothrix isolated in this case is more closely related culturally and morphologically to Streptothrix putorii than to Streptothrix muris-ratti. Morphologically, it differs from the Streptothrix putorii in being shorter, as a rule. It differs culturally in producing a grayish color rather than a grayish-yellow color on dextrose-blood agar, in growing less abundantly on plain and dextrose agar, and in fermenting dextrose, raffinose, maltose and leontose.

The chief points of interest in this case of rat-bite fever are the presence of a streptothrix in the polymorphonuclear leukocytes during life; in blood cultures after death; in smears of bone marrow, and possibly in the ganglion cells near the suprarenals.

EXPLANATION OF PLATE 5

Fig. 1.—Blood smear made during life; carbol-thionin and carbol-gentian-violet; × 1,000.

Fig. 2.—Twenty-hour-growth in ascitic broth of streptothrix isolated from blood after death. Carbol-gentian-violet, × 1,000.

Fig. 3.—Section through ganglion near suprarenal, Levaditi preparation. × 800.

Table 5

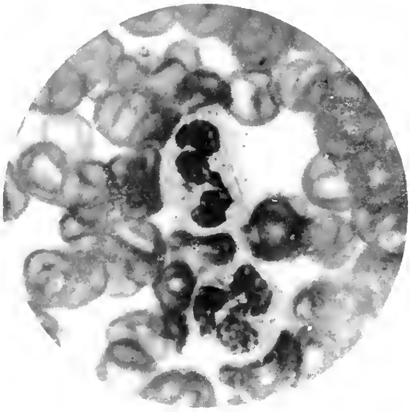


Figure 1

Figure 2

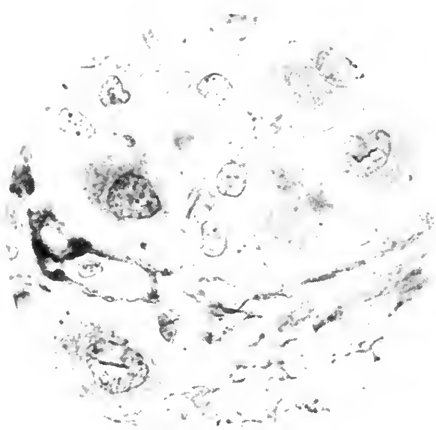


Figure 3

FURTHER OBSERVATIONS ON HEMOLYTIC STREPTOCOCCI IN MILK

David John Davis

From the Department of Pathology and Bacteriology, University of Illinois, College of Medicine, Chicago

In a previous paper[1] I presented data obtained from the analysis of milk samples from nine different dairies in the city of Chicago. Of 328 specimens examined hemolytic streptococci were found in 85. Forty-five of the 328 specimens were certified milk, the remainder being pasteurized, some by the flash method, others by the holding process. Forty per cent. of the certified samples yielded hemolytic streptococci. The number of pasteurized samples yielding hemolytic streptococci varied greatly among the different dairies. Sixteen samples from one yielded none. The others gave positive results varying from 6-45% of the samples examined. A discussion of the heat resistance, virulence and other properties of these streptococci are given in the paper. No definitely virulent organisms were met with though a number of strains in moderate doses caused lesions in the joints of animals.

Since then further examination of samples of both certified and pasteurized milk from a certain dairy indicated the presence of hemolytic streptococci commonly in the former, very rarely in the latter. A more systematic study, therefore, has been made of both certified and pasteurized samples from the dairy for the presence of this type of streptococci. It should be stated that the certified milk and the pasteurize milk came to the distributing dairy from entirely different sources. The difference between them therefore may not be merely one of pasteurization.

The examinations were made during January, February and March. The milk coming to the laboratory was plated as soon as possible and, in case there was delay, the specimens were placed in a good refrigerator. Definite amounts were measured in sterile pipets and agar plates made. These were then incubated and the results recorded after 48 hours.

Ninety-two samples, 46 pasteurized and 46 certified, were examined. Total bacterial counts were made in 20 of each. The plate counts in the certified specimens ranged from 2,000-15,000 per c c. those in the pasteurized specimens from 10,000-45,000 per c c.

Received for publication June 26, 1918.

[1] Jour. Infect. Dis., 1916, 19, p. 236.

Hemolytic streptococci (colonies surrounded by a clear, wide zone of hemolysis) were isolated in one of the 46 pasteurized specimens, their number in this instance being 20 per c c of milk. Of the 46 certified samples, 22 yielded hemolytic streptococci on the blood plates, their number ranging from 8-2,400 per c c of milk.

These strains of streptococci were all practically alike in their hemolytic action, producing clear zones from 1-3 mm. across. The colonies themselves were small and gray. In plain broth they usually grew well and caused an early turbidity which later cleared by sedimentation. They all rapidly acidified and coagulated milk in 24 hours. The milk became first pink, then, especially at the bottom of the tube, very pale; later only little color remained. Morphologically the cocci appeared in pairs or short chains. In fluid mediums they at times tended to form long chains, there being considerable variation in this respect. The cocci, as a rule, are slightly elongated, Gram-positive and not encapsulated.

Twelve of the most typical strains were grown for 24 hours in serum broth (1-4), 2 c c of which was inoculated into the ear veins of young rabbits of approximately 1,000 gm. With this dosage 2 of the strains were pathogenic for the rabbits. One strain (C-16) after several days caused an infection of the left knee which at the end of 2 weeks was large and fluctuating and on aspiration yielded the streptococci. No other lesions appeared and the animal lived several weeks without further manifestations. The other strain (C-19) after 3 days caused an involvement of the left wrist and right knee joint and death 5 days after the injection. There was congestion with numerous hemorrhages in the cecum, also small hemorrhages in the endocardium and the chordae tendineae of the right heart. Definite endocarditis was not present. There was marked joint and tendon involvement of the extremities, especially the left fore and right hind foot. Small hemorrhages appeared at various points along the spinal cord and also in the meninges. From various joints, the heart blood and spinal cord pure cultures of the hemolytic streptococci were grown.

A few drops of a 24-hour ascitic-broth culture of the same organism was introduced intraperitoneally in a white mouse, which died 4 days later of a streptococcic septicemia. Four other strains injected similarly into white mice produced no effect.

It is evident that this strain was decidedly more virulent for animals than the other strains of the series, though in milk and otherwise it was quite like them. The other strains in the dosage stated produced no lesions, and even in considerable larger doses were quite without virulent properties. It is to be noted that in large doses (5-15 c c ascites-broth culture) given intravenously in young rabbits, these hemolytic streptococci invariably localize in various tissues and cause local lesions. The joints are a common site of infection as is also the region of the cecum.

All the streptococci described apparently belong to the hemolytic streptococci of bovine origin which I described in my previous paper. They are not as a rule virulent for animals as are those of human origin and on milk they behave quite like streptococcus lacticus. Indeed, excepting the hemolytic property they are practically identical with this organism which usually reveals a definite green coloration on blood without appreciable hemolysis. My observations, as pointed

out before, therefore indicate that there are both hemolytic and nonhemolytic lacticus strains both of which are nonvirulent, active acidifiers and coagulators of milk.

The hemolytic streptococci so often found in milk, especially nonpasteurized, are readily distinguished from the human varieties by their behavior in milk. But it should be pointed out that occasionally in milk occur varieties of hemolytic cocci quite like the human. Such sometimes occur in the udders and may be the cause of epidemics of sore throat. Probably not all strains of such organisms are capable of causing such epidemics any more than are all strains of streptococci of different human origins. At any rate hemolytic streptococci are found in milk from diseased udders which are quite different from the hemolytic milk streptococci of the lacticus type.

I may repeat what I have emphasized[1] that in milk there are strongly hemolytic streptococci of the lacticus type which are probably of no pathogenic significance to man, and hemolytic streptococci quite like the human varieties and which may or may not be highly pathogenic for man. They are probably directly or indirectly human in origin.

SUMMARY

Further observations have revealed the presence of hemolytic streptococci of the lacticus type in dairy milk in 23 of 92 samples.

In this series they were far more common in nonpasteurized than in pasteurized milk.

They are less virulent for rabbits than the hemolytic streptococci of human origin. Two strains were found with moderate pathogenic power for rabbits.

While, in general, one may be practically sure that organisms of the lacticus type are not dangerous to man, still individual organisms or strains of human, milk or bovine origin suspected of being responsible for sore throats or other infections in man should be carefully studied and compared with a view to finding specific common characters.

THE STREPTOCOCCI OF THE ACTINOMYCES-LIKE GRANULES OF THE TONSILS

I. Pilot and D. J. Davis

From the Department of Pathology and Bacteriology, University of Illinois, College of Medicine, Chicago

In 1914 one[1] of us called attention to the nature of the actinomyces-like masses that are commonly found in the crypts of the faucial tonsils. At that time a series of 122 pairs of tonsils were carefully examined for the presence of these bodies which were found in 30. Now a larger series has been examined and certain new facts have been gathered with reference to the nature of the streptococci found in them.

The granules are grayish or yellowish bodies, rather firm, and on microscopic examination have a raylike structure resembling the sulphur granules of actinomycosis for which they have been mistaken. Their size is variable. They are usually about the size of a pinhead, but not infrequently larger. In one instance, a granule measured 15 mm. in all dimensions. They may be so small as to be visible only with the aid of a hand lens or microscope. In the crypts of the tonsils, they lie free either near the base or nearer the orifice through which they may protrude. They are quite brittle and when disintegrated have a foul and disagreeable odor.

The frequency of their occurrence is difficult to estimate. Careful examination of smears from the crypts of almost all tonsils will reveal such masses in minute form. As stated, in the first series the granules were visible to the naked eye in 30 of 122 pairs of tonsils. In the present series they were found in 31 of 135 pairs. If they are present, there is usually more than one. If single it is often large. Small granules occur in numbers of 3 or 4 or more. In one instance as many as 20 were found. During the process of enucleation of the tonsils many are frequently lost.

Microscopically they have a raylike structure made up of filaments, and at the periphery of brushlike structures which consist of a central shaft of filaments about which are arranged large numbers of bacilli, spirilla and cocci. The bacilli are perpendicular to the central shaft.

Received for publication June 26, 1918.

[1] Davis: Jour. Infect. Dis., 1914, 14, p. 144.

They are anaerobic organisms, and resemble in their morphologic and cultural characteristics the various forms of the fusiformis group.

In the present study particular attention is directed to the streptococci associated with the filamentous forms. In smear preparations from the crushed granules, they appear in pairs, less often singly or in short chains. They intermingle freely in variable numbers with the bacilli and spirilla, and do not seem to have any characteristic distribution. In some granules the cocci are very few in number, while in others they are the predominating organisms. They are strongly gram-positive.

For isolation of these cocci the granules were collected immediately after tonsillectomy from patients, chiefly children, in the ages of 5-16. The granule was first washed several times in sterile salt solution and then crushed by a sterile knife. Plain agar with a reaction of +1 to which defibrinated human blood was added in the proportion of 1:20, was inoculated with a loopful of material, and then poured into Petri dishes. Three dilutions were made. The plates were then incubated under both aerobic and anaerobic conditions. To remove the oxygen, an alkaline pyrogallic acid mixture was placed in the bottom of a desiccating jar. The plates were placed above, and the jar was firmly sealed to exclude the air. The anaerobic plates were incubated for 48 hours.

Two kinds of streptococcus colonies would develop in both aerobic and anaerobic plates. There were small colonies surrounded by clear zones of hemolysis measuring in width from 3-5 mm.; the other type was surrounded by a green or grayish-green zone. The proportion in which these colonies occurred, in the aerobic and anaerobic plates, is shown in Table 1. We see that the colonies on the anaerobic plates are generally more numerous than those on the aerobic plates; that the viridans type is constant, while the hemolytic type may or may not be present. Although the number of hemolytic colonies are more numerous on the anaerobic plates, the growths on the aerobic and anaerobic subcultures are equally profuse. In the case of the viridans type, there may be no growth on the aerobic plates and no growth or a very fine growth on the first aerobic subcultures. In the subsequent subcultures the growth becomes gradually heavier until the third or fourth transfers when the growth is equally heavy in the aerobic and anaerobic slants. The anaerobic subcultures were made on blood-agar slants using the Wright alkali-pyrogallic acid method.

Tall tubes of ascitic dextrose agar were inoculated with 22 strains. In all instances both the hemolytic and viridans types preferred the middle portion of the column, with no growth within 1 cm. of the surface, and little or no growth at the bottom of the tube. Those colonies which grew better anaerobically showed a tendency to grow more luxuriantly in the deeper portions of the tubes. In ascitic dextrose broth these organisms produced a granular deposit on the bottom and sides of the tubes. Some caused a slight or moderate, diffuse turbidity of the medium. Smears from the broth cultures of the hemolytic type, revealed medium-sized or long chains, rarely short chains, and short, sometimes medium-sized chains in cultures of the viridans type. The cocci of the strongly anaerobic strains were considerably smaller in size than those of the indifferent strains.

Four strains of strongly anaerobic cocci which showed small forms in smears were tested for filtrability. An anaerobic blood-agar slant was washed with

TABLE 1

Showing the Number and Character of Streptococci Found in the Granules

Number	Hemolytic Streptococci		Streptococcus Viridans		Growth on 1st Subculture				Growth on 2nd Subculture				Growth on 3rd Subculture			
	Aerobic	Anaerobic	Aerobic	Anaerobic	Hemolytic		Viridans		Hemolytic		Viridans		Hemolytic		, Viridans	
	Number of Colonies	Number of Colonies	Number of Colonies	Number of Colonies	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
1	1	1	1	800	+++	+++	0	+++	—	—	+	+++	—	—	++	+++
2	37	100	0	10	+++	+++	+	+++	—	—	++	+++	—	—	+++	+++
3	2	100	0	100	+++	+++	0	+++	—	—	+	+++	—	—	++	+++
4	30	0	200	200	+++	+++	+	+++	—	—	++	+++	—	—	+++	+++
5	0	0	50	50	—	—	++	+++	—	—	+++	+++	—	—	+++	+++
6	0	0	0	30	—	—	+	+++	—	—	++	+++	—	—	+++	+++
7	0	200	2	200	+++	+++	+	+++	—	—	++	+++	—	—	+++	+++
8	0	0	100	200	—	—	+	+++	—	—	+++	+++	—	—	+++	+++
9	1	10	25	50	+++	+++	+++	+++	—	—	+++	+++	—	—	+++	+++
10	2	100	2	500	+++	+++	++	+++	—	—	+++	+++	—	—	+++	+++
11	0	0	0	15	—	—	+	+++	—	—	++	+++	—	—	+++	+++

sterile salt solution, and the suspension was filtered slowly through a tested Berkefeld filter. Aerobic and anaerobic cultures of the filtrate were sterile in all instances.

Cultures were made directly from crypts in which the granules were found, in order to compare the streptococcal flora on the crypt epithelium with that of the granules. In Table 2 are given the results of cultures from 4 pairs of tonsils. In all instances the number of colonies on the aerobic and anaerobic plates were about the same. The hemolytic colonies were considerably greater than those of the viridans type. In the granules, it will be recalled, the growth on the anaerobic plates was more profuse and the number of the viridans type of streptococcus exceeded the hemolytic.

Fermentation tests were made with the strains isolated from the granules and from the crypts—22 in all. Litmus ascitic carbohydrate broth was employed. Table 3 shows the results.

TABLE 2

Showing the Number and Character of Streptococci Found on Crypt Epithelium

			Number of Colonies	
			Aerobes	Anaerobes
1	Right tonsil	Hemolytic	500	20
		Viridans	10	4
	Left tonsil	Hemolytic	200	50
		Viridans	8	6
2	Right tonsil	Hemolytic	20	20
		Viridans	0	1
	Left tonsil	Hemolytic	30	30
		Viridans	10	10
3	Right tonsil	Hemolytic	50	30
		Viridans	0	0
	Left tonsil	Hemolytic	100	50
		Viridans	0	0
4	Right tonsil	Hemolytic	150	150
		Viridans	100	100
	Left tonsil	Hemolytic	—	—
		Viridans	—	—

All strains fermented lactose and saccharose; none fermented inulin and mannite. According to the classification of Holman,[2] the hemolytic strains which fermented salicin would fall in the Streptococcus pyogenes group, while those that did not, into the anginosus group. Of the strains here examined there are 5 of the former and 6 of the latter. The viridans strains which fermented salicin belong in the Streptococcus mitis group, while those that did not ferment salicin belong in the Streptococcus salivarius group. In this series, of the former there are 4 and of the latter 7.

Ten strains, 4 hemolytic and 6 viridans, were tested for their pathogenicity in rabbits. The hemolytic form of streptococci were injected intravenously in 2 c c doses of a 48-hour ascitic-dextrose-broth culture. There occurred in all a slight loss of weight, and rise of temperature, followed by rapid swelling of several joints. On postmortem the lesions were those of multiple arthritis, and

[2] Jour. Med. Research, 1916, 34, p. 377.

cultures gave organisms similar to those injected. The viridans forms of streptococci were far less virulent. The centrifugalized sediment of 10-15 c c of ascitic dextrose broth was necessary to produce lesions. The lesions were chiefly of the joints and heart, multiple arthritis, endocarditis, pericarditis being the chief ones. The strongly anaerobic strains did not differ from the others in their pathogenic properties.

TABLE 3

Fermentation Tests of Streptococci Isolated from Granules and from Crypt Epithelium

No.	Origin	Type	Dextrose	Salicin	Mannite	Maltose	Lactose	Inulin	Saccharose	Raffinose
1	Granules	Hemolytic	+	0	0	+	+	0	+	0
2	Granules	Hemolytic	+	0	0	+	+	0	+	0
3	Granules	Hemolytic	+	+	0	+	+	0	+	0
4	Granules	Hemolytic	+	0	0	0	+	0	+	0
5	Granules	Hemolytic	+	0	0	+	+	0	+	0
6	Granules	Hemolytic	+	+	0	+	+	0	+	+
7	Granules	Hemolytic	+	+	0	+	+	0	+	0
8	Crypts	Hemolytic	+	0	0	+	+	0	+	0
9	Crypts	Hemolytic	+	0	0	+	+	0	+	0
10	Crypts	Hemolytic	+	+	0	+	+	0	+	0
11	Crypts	Hemolytic	+	+	0	+	+	0	+	0
12	Granules	Viridans	+	0	0	+	+	0	+	0
13	Granules	Viridans	+	+	0	+	+	0	+	+
14	Granules	Viridans	+	0	0	0	+	0	+	+
15	Granules	Viridans	+	+	0	+	+	0	+	+
16	Granules	Viridans	+	0	0	+	+	0	+	0
17	Granules	Viridans	+	+	0	+	+	0	+	0
18	Granules	Viridans	+	0	0	+	+	0	+	+
19	Granules	Viridans	+	0	0	+	+	0	+	0
20	Granules	Viridans	+	0	0	+	+	0	+	+
21	Granules	Viridans	+	0	0	+	+	0	+	0
22	Crypts	Viridans	+	+	0	+	+	0	+	+

The occurrence of streptococci with strong anaerobic properties has been noted by several investigators.

Von Gottlieb Salus[3] discusses the frequency of obligate anaerobic streptococci particularly in connection with infections of the female pelvic organs. Special reference is made to a group of anaerobic streptococci termed Streptococcus putridus. It is still an open question whether or not this group consists of organisms of a strictly anaerobic type, or is derived and can be classed under the types included under the name of Streptococcus pyogenes. Warren and Herrick[4] in tabulating 134 cases of bacteremia, observed three instances of anaerobic streptococci in cases of pelvic infections. Curtis[5] isolated an anaerobic streptococcus from a fibroid in one instance. Lingelsheim[6] states that occasionally streptococci occur which on first cultivation require anaerobic conditions, but subsequently may become adapted to aerobic conditions.

[3] Centralbl. f. Bakteriol., O, I, 1916, 77, p. 1.

[4] Am. Jour. Med. Sc., 1916, 151, p. 556.

[5] Jour. Infect. Dis., 1916, 19, p. 712.

[6] Handbuch der Path. Mikroorganismen, 1912, 4, p. 453.

Streptococci of the type isolated in the present series have been cultured from other parts of the body, together with bacilli and spirilla, usually of the fusiform type. Kroening[7] isolated an anaerobic streptococcus in symbiosis with a short curved bacillus. The streptococcus was not pathogenic for animals. Dick[8] in two cases noted the occurrence of Streptococcus viridans associated with Bacillus fusiformis, growing better anaerobically than aerobically. Rosenow[9] found strongly anaerobic streptococci together with fusiform bacilli in 2 cases of appendicitis. Koessler and Moody[10] mention the occurrence of a very small streptococcus with gram-negative fusiform bacilli all anaerobic in the bronchial exudate of patients suffering from bronchial asthma.

The occurrence in the tonsillar crypts of streptococci with strong anaerobic properties, associated with the more strictly anaerobic organism, the Bacillus fusiformis, is interesting and may be another instance of the adaptive property of the streptococcus to its environment.

There is no very clear evidence at present of the pathogenicity of these granules in the tonsils but it may be pointed out that here are foci where organisms with potential pathogenic powers are commonly found and apparently protected. The numerous instances of infectious processes now known caused by hemolytic streptococci or in which streptococci and bacilli of the fusiform types are associated make it necessary to carefully consider the possibility of danger lurking in these foci under special conditions.

CONCLUSIONS

Two types of streptococci, the hemolytic and the viridans, were isolated from the actinomyces-like granules of the tonsils.

The viridans type showed strong anaerobic properties. The hemolytic cocci did not.

Otherwise, the streptococci in their cultural and morphologic characteristics and pathogenic properties resemble streptococci from various parts of the mouth and from the crypts of normal and diseased tonsils.

According to the classification of Holman the hemolytic organisms isolated from the granules are of two varieties, Streptococcus pyogenes and Streptococcus anginosus; the viridans types also are of two varieties, Streptococcus mitis and Streptococcus salivarius.

7 Centralbl. f. Gynäk., 1895, 16, p. 409.
8 Jour. Infect. Dis., 1913, 12, p. 191.
9 Jour. Infect. Dis., 1915, 16, p. 240.
10 Tr. Clin. Soc. Int. Med., 1917, 1, p. 7.

A CHROMOGENIC BACILLUS FROM A CASE OF ROUP

B. F. Kaupp

From the North Carolina Agricultural Experiment Station, West Raleigh, N. C.

A buff plymouth rock cock, 2 years old and a member of the station and college flock, was apparently in good health till about 3 weeks previous to his death. The bird became dull, thinner in flesh, the comb dark and somewhat shriveled and there was difficult breathing and a general unkempt appearance. On opening the mouth there was noted a mass of diphtheric material formed from a wide-spread oral diphtheric area. The nasal passages were partially occluded with a thin watery discharge. Breathing was partially oral.

At the necropsy the following observations were made: There are no evidences of disease except in the region of the nostrils and mouth. There is an offensive roupy odor from the mouth. In the mouth cavity is observed a wide-spread ulcerated area covered with a yellowish-white membrane, diphtheric in nature. There is a mucopurulent accumulation in the nasal passages and in the trachea.

Microscopically the kidneys show both active and passive congestion. The cells of the tubules are in a state of cloudy swelling. Many nuclei of the cells of the convoluted tubules are in a state of karyorrhexis, other cells show a total disappearance of the nuclei. The lumen of many of the convoluted tubules are obliterated and the cell bases have moved in from their peripheral positions.

Smears from the lower third of the trachea showed apparently almost a pure culture of a rather short thick organism about two to three times as long as broad. From the purulent tracheal material, made in suspension in salt solution, there was inoculated intraperitoneally into a half-grown rabbit, 4 c c. The rabbit died of septicemia in 20 hours. Pure cultures of an organism, which has been designated as 3a, were isolated. These pure cultures were obtained from the heart blood, liver, and peritoneal fluid.

A second rabbit was inoculated with 1 c c of an emulsion made from the culture of the heart blood of Rabbit 1. In preparing the emulsion just as much culture was used as would adhere to one-fourth inch space of the platinum needle. This injection was made intraperitoneally. The rabbit died of septicemia in 18 hours. Pure cultures were obtained from the heart blood, liver, and the peritoneal fluid.

Character of Organism 3a.—Bacillus on artificial medium. Rather coccoid in form from heart blood of animal which has died of septicemia from this organism. In later transfers the organism is distinctly bacillary in form.

Cultures 5 days old show organisms averaging 0.49 by 2.3 microns and from the heart blood of the rabbit 0.43 by 0.64 microns.

Ends rounded.

No spore formation.

Flagella, peritrichiate.

Capsule, absent.

Growth at 37 C., abundant.

Received for publication June 27, 1918.

Staining reactions, readily with ordinary anilin dyes as fuchsin, methylene blue, gentian violet, Loeffler's methylene blue.

Reaction to Gram stain, negative.

Cultural Features.—Agar stroke, growth, moderate. Form of growth, echinulate. Elevation, raised. Luster, glistening. Topography, surface smooth, porcelain-like. Optical characters, opaque. Chromogenesis, green, varying from a turtle green at the end of 2 days to a shamrock green in old cultures. Comparisons made with Ridgway's color chart.

Potato.—Growth, scanty. Form of growth, spreading. Elevation, flat. Luster, dull. Topography, contoured. Chromogenesis, brownish with a tendency to green later. Odor, decided. Consistency, butyrous. Medium, browned. Old cultures, brownish-green. The diastatic action was feeble.

Agar Stab.—Growth, abundant. Best on top. Surface growth, abundant. Line of puncture, filiform.

Gelatin Stab.—Growth, best on top. Line of puncture, filiform. Liquefaction, stratiform. Medium, greened.

Nutrient Broth.—Surface growth, skumlike. At times scanty with pellicle. Clouding, at first moderate, later strong. Odor, decided. Sediment, granular—on agitation abundant.

Milk.—Clearing without coagulation. Digested medium gradually becomes greenish.

Agar Colonies.—Dew drop colonies. Colonies at the end of 24 hours are smooth but in older colonies the culture has a tendency to become grumous.

Growth in Broth Over Chloroform.—At first feeble, but after a few days there had developed a moderate growth. Chloroform restrains growth.

Growth in Glycerol Broth.—The organism grew abundantly but without gas or acid formation.

Reduction of Nitrates.—At the end of 14 days there was a marked reduction of nitrates.

Toleration of Acids.—Fuller's scale:

Acid	24 hours	48 hours	72 hours	7 days
+ 10*	+	+	+	+
+ 20	—	—	—	—
+ 30	—	—	—	—
+ 40	—	—	—	—
+ 50	—	—	—	—

* Control tube.

There was no growth in any of the tubes. The control showed abundant growth.

Toleration to NaOH.—Fuller's scale:

NaOH	24 hours	48 hours	72 hours	7 days
— 10*	+	+	+	+
0	+	+	+	+
+ 10	+	+	+	+
+ 20	—	+	+	+
+ 30	—	—	+	+
+ 40	—	—	—	+
+ 50	—	—	—	—
+ 60	—	—	—	—

* Control tube.

Plus 50 completely arrested growth; 40 showed slight growth at the end of 7 days, and 30 at the end of 72 hours.

The vitality of the culture is long continued on artificial medium. The optimum temperature for growth is 37 C.

Test Against Phenol.

Percentage in tube	24 hours	48 hours	72 hours	96 hours	7 days
control	+	+	+	+	+
0.1	+	+	+	+	+
0.25	—	+	+	+	+
0.5	—	—	—	—	—
0.75	—	—	—	—	—
1.0	—	—	—	—	—
2.0	—	—	—	—	—
3.0	—	—	—	—	—
4.0	—	—	—	—	—
5.0	—	—	—	—	—

Phenol, 0.25%, arrested growth and 0.5% killed the organisms.

Thermal Death Point.—Time of exposure in water bath, 10 minutes.

Temperature, C.	24 hours	48 hours	72 hours	96 hours	7 days
control	+	+	+	+	+
50	+	+	+	+	+
55	—	+	+	+	+
60	—	—	—	—	—
65	—	—	—	—	—
70	—	—	—	—	—
75	—	—	—	—	—

The exposure to 55 C. arrests growth and 60 C. kills the organisms.

Test tubes containing 10 c c each of broth were inoculated with Culture 3a and the surface covered with autoclaved oil of petrol. The results were negative though the cultures were incubated 10 days.

Below is given the results of the action of this organism on 6 different sugars. All fermentation tubes contained 1% of the sugar. Titrations made at the end of 14 days.

Sugar	Control	Tube 1	Tube 2
Saccharose	+1	+0.5	+0.5
Lactose	+1	+0.5	+0.5
Dextrose	+1	+1.0	+1.0
Maltose	+1	+0.5	+0.5
Raffinose	+1	+0.5	+0.5
Mannite	+1	+1.0	+1.0

There was no change in the dextrose and mannite. There was slight alkali formation in the saccharose, lactose, maltose and raffinose.

Pathogenicity.—In the early part of the paper the pathogenicity to rabbits has been shown. Several birds were inoculated. The following average result will serve to show the effect on the fowl. Inoculations were made in the infraorbital sinus, nostrils, skin of the face, intratracheally, comb, wattles, mouth, and eye. The surface inoculations were made intradermally. At the end of 24 hours there was noted at the point of inoculation, of the comb and wattles, considerable swelling. In the mouth there was a white ulceration of the mucous membrane at the point of inoculation. This ulcer showed little tendency to spread and healed by the end of the 5th day. The tumefaction of the comb attained the size of a half corn grain, some ulcerating and others subsiding after a few days without ulceration. Some birds showed more

resistance than others. Half-grown single comb white leghorn cockerels were used to ensure none having gained artificial immunity, as in this section most flocks, sooner or later, go through at least a mild attack of chickenpox or roup. In those birds in which ulcers developed there was present depression, weakness, partial loss of appetite, lopped comb and a rapid loss of weight. In those cockerels in which ulceration did not take place and in which the swelling soon subsided these symptoms were not noticed. In those cases in which ulceration took place recovery occurred in from 10-14 days. The nasal, intratracheal and infraorbital inoculations on the uninjured mucous membranes were all negative.

DISCUSSION

In the study of roup at this station there has been isolated an organism which, so far as our literature goes, perhaps has not hitherto been described. The organism is a rather short, thin bacillus measuring, on an average, 0.43 by 0.64 mikrons. The ends are rounded. It is peritrichiate, hence motile. It does not possess capsule. It grows abundantly at 37 C. and produces a greenish pigment. It is gram-negative and stains readily with ordinary anilin dyes. It is aerobic, growing very little along the needle track and when the oxygen is entirely excluded it does not grow. It reduces nitrates; 2% normal HCl completely arrests its development; 2% NaOH arrests but does not completely retard its growth as at the end of 48 hours feeble development was noted in all tubes with this percentage; 5% completely arrested its growth; 0.25% phenol retarded its growth and 0.5% killed it; 55 C. retarded its growth and 60 C. killed it.

There was no change in dextrose and mannite broth nor any gas formation. There was a slight formation of alkali in saccharose, lactose, maltose and raffinose. There was no gas formation.

The organism is highly pathogenic in rabbits, killing of septicemia in 18-20 hours, and shows some pathogenic properties for the fowl when injected into injured tissues as in intradermal inoculations.

In terms of the numerical system of recording the salient characters of an organism (group number), as suggested by the Society of American Bacteriologists, we have as a total 211.3331423.

SUMMARY

There is here given an account of a chromogenic bacillus found in connection with roup and chickenpox probably not before reported.

The organism described is highly pathogenic for rabbits and shows some pathogenic properties for the tissues of the fowl.

IMMUNE REACTIONS WITH DIPLOCOCCI ISOLATED FROM MEASLES AND RUBELLA

RUTH TUNNICLIFF AND MARY WILMARTH BROWN

From the John McCormick Institute for Infectious Diseases, Chicago

Tunnicliff[1] has described gram-positive cocci which were isolated in anaerobic cultures from the blood of measles and rubella patients. The measles coccus is small and round, the rubella coccus is larger, elongated, with pointed ends and a capsule. Both generally occur in pairs. Diplococci similar to those isolated from the blood were also cultivated from the throat, nose, eye and ear of measles patients, and from the throat of rubella patients. Immune reactions in patients with measles and rubella were studied and specific opsonins for the measles and rubella diplococci were found to develop during the course of measles and rubella, respectively, disappearing with recovery. There were no changes in the opsonic content of the blood in either disease for the Streptococcus viridans, pneumococcus or the poliomyelitis coccus in any of the serums examined. Agglutination experiments were unsatisfactory on account of the tendency of the cocci to agglutinate spontaneously.

COMPLEMENT FIXATION

A few complement fixation tests have been made with measles and rubella serum using as a rule a suspension of the diplococci isolated from the blood of measles patients, but also a mixture of strains, as the measles antigen, and a mixture of strains isolated from the blood and throat of rubella patients for the rubella antigen.

The cocci for the fixation tests were grown for 24 hours in ascitic dextrose broth (0.2% dextrose, 0.6% acid to phenolphthalein, 10% ascitic fluid), removed by centrifugation, washed and suspended in sterile normal salt solution. The mixtures were then heated at 56 C. for 30 minutes.

The antisheep-rabbit system was used in one-tenth the volume of the original Wassermann test. The anticomplementary unit of antigen was determined, and the hemolytic system was standardized by an amboceptor titration. In the test, the serum remained constant at 0.02 c c; the antigen varied from $\frac{1}{4}$-$\frac{1}{20}$ of the anticomplementary unit. Fresh guinea-pig serum, 0.1 c c of a 1:10 dilution (2 units), was used as a complement. Serum, antigen, and comple-

[1] Jour. Am. Med. Assn., 1917, 68, p. 1028; Jour. Infect. Dis., 1918, 22, p. 462.

ment were incubated at 37 C. for 1 hour. Two units of previously titrated antisheep amboceptor and 0.1 c c of sheep corpuscles in 5% suspension were added to each tube, and the whole incubated for 30 minutes. The customary controls (antigen, serum and hemolytic system) were set up with each test.

Fifteen cases of measles were examined, 8 before the 8th day; the others during the 2nd and 3rd weeks following the infection. The 8 early cases showed some fixation on 1 day, but were examined too infrequently to determine the duration. Five patients were examined twice and not on successive days, 1 was examined only once, and 1 every 3rd day for 12 days. One fixed with the homologus blood coccus, 6 with an heterologous blood coccus, 3 of these fixed also with the mixed measles antigen, and 1 with the mixed antigen only. The fixation was strong in 1 case only when inhibition occurred in 1/32 of the anticomplementary unit, in 4 inhibition occurred in 1/16 of the anticomplementary unit, and 3 in 1/4 of the anticomplementary unit. The serum of 3 patients also caused inhibition in 1/4 of the anticomplementary unit, of the rubella antigen and a hemolytic streptococcus (scarlet fever gland) antigen.

Of the 13 rubella serums, only 7 were examined more than once, and then only every other day for 3 or 4 days. They were all studied during the week of the attack. Only 5 of the 13 serums showed any fixation for the rubella antigen and then only to a slight degree (1/4 the anticomplementary unit) and none for the measles antigen. Nine serums were tested with hemolytic streptococcus antigen and 4 showed strong fixation (1/32 to 1/64 the anticomplementary unit) and 4 slight fixation. When the patient's serum was examined more than once the positive fixation with the hemolytic streptococcus antigen was found to persist. One of the patients showing strong fixation, had both scarlet fever and rubella, and hemolytic streptococci were present in the throat in some of the other rubella patients examined at the end of the attack.

The results indicate that the serum of measles and rubella patients contain complement fixing bodies for their respective diplococci to a slight degree early in the disease at about the same time the increase in opsonins occurs.

IMMUNE REACTIONS IN RABBITS INJECTED WITH COCCI FROM MEASLES AND RUBELLA

On account of the frequent presence of nonhemolytic diplococci and streptococci in normal and pathologic conditions and the difficulty of differentiating them by their cultural and morphologic characteristics (especially after cultivation), an attempt has been made to differentiate the cocci isolated from measles and rubella from each other and from similar cocci, by immunological methods.

Howell[2] immunized rabbits with some of these measles and rubella diplococci as well as with nonhemolytic and hemolytic streptococci, and concluded that the positive complement fixations could not be grouped in any way that would justify a classification of streptococci based on the complement fixation test.

[2] Jour. Infect. Dis., 1918, 22, p. 230.

The experiments in this work were therefore confined to agglutination and opsonic determinations.

Rosenow and Gray[3] found agglutination tests useful in differentiating the poliomyelitis streptococcus from streptococci and pneumococci from other sources. Mathers and Tunnicliff[4] found that specific opsonins for the poliomyelitis coccus develop during the course of acute poliomyelitis, and in conjunction with Howell[5] that the serum of rabbits immunized against different strains of poliomyelitis cocci contain opsonins in high concentrations, which were apparently specific for the poliomyelitis cocci. Agglutinins were demonstrable in low dilutions only, and spontaneous agglutination of the cocci interfered with the tests. Nuzum and Willy[6] have found that by means of opsonic determinations of immune horse serum, they were able in 20 instances to identify and separate strains of poliomyelitic cocci from other strains of cocci presenting more or less confusing cultural and morphologic similarities. The point of opsonic extension of the immune horse serum reached as high as 1 : 6,000.

Rabbits, 3-6 months old, were first inoculated intravenously with 10 billion killed diplococci—the measles rabbits with a mixture of 4 strains, 1 blood, 1 eye, 1 ear and 1 throat; the rubella rabbits with a mixture of 2 blood and 2 throat strains. Four days later they were inoculated intravenously with ½ the washings in salt solution of the growth of each organism on 1 blood-agar slant. The dose was increased every 4 days until 7 injections were received, when the dose was reduced to the original dose of living cocci given every 10 days or 2 weeks to maintain the antibody content. The measles cocci, although 3 of the strains were more than a year old, frequently made the rabbits very ill, in which case the dose was diminished and the length of time between injections increased. There was good antibody production after the third injection.

Sixty-five strains of diplococci and streptococci have been examined, 8 from measles, 25 from rubella, and 32 from other sources. The measles and rubella cocci and 28 cocci from other sources were green-producers when first isolated. Four strains were hemolytic streptococci. In the meantime 1 of the measles strains (ear) became more or less hemolytic and 2 of the rubella blood strains lost their power to produce green on blood-agar plates to a large extent, but were still nonhemolytic.

AGGLUTININS

Unheated serum was used in varying dilutions, using the serum of a normal rabbit as control. The experiments were made in capillary pipets in which agglutination is readily observed. The cocci from measles when first isolated agglutinated spontaneously so that no satisfactory tests could be made, but after long isolation they grew diffusely in 1% dextrose and sometimes in calcium carbonate broth and satisfactory agglutination tests could be made. Some of the rubella strains produced good suspensions; the majority were impossible to work with on account of spontaneous clumping. The specimens were incubated at 35 C. for 2 hours and placed in the refrigerator for 20 hours at which time the readings were made.

[3] Jour. Infect. Dis., 1918, 22, p. 345.
[4] Jour. Am. Med. Assn., 1916, 67, p. 1935.
[5] Jour. Infect. Dis., 1917, 21, p. 292.
[6] Jour. Infect. Dis., 1918, 22, p. 258.

The serum of the immune measles rabbit agglutinated markedly, both the homologous and the heterologous measles strains (Table 1), and also, in low dilutions, 3 rubella strains and 1 coccus from a case of acute bronchitis.

The rubella rabbit serum agglutinated 3 of the homologous strains, the other strain agglutinating spontaneously, but only 1 of the heterologous strains and to a slight extent also a poliomyelitis strain, so that although the majority of the rubella strains agglutinated spontaneously, those that did not do so were generally not agglutinated by the rubella immune rabbit serum.

TABLE 1

AGGLUTINATION TESTS

Sources of Cocci	Dilutions of Serum													
	Serum of Rabbit Immunized With Measles Coccus								Serum of Rabbit Immunized With Rubella Coccus					Normal Rabbit Serum
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:10	1:20	1:40	1:80	1:160	1:10
From blood[1] in measles...	++	++	++	++	0				0					0
From blood in measles....	++	++	++	+	0				0					0
From eye[1] in measles......	++	++	0											
From ear[1] in measles......	++	++	++	++	++	+	0		0					0
From throat in measles...	++	++	++	+	0				0					0
From throat in measles...	++	++	++	++	++	++	+	0	0					
From throat in measles...	++	++	++	++	++	++	++	++	0					0
From nose in measles......	++	++	++	0					0					0
From blood[1] in rubella....	0								++	++	0			0
From blood[1] in rubella....	0								++	++	++	++	0	0
From throat[1] in rubella...	0								++	++	++	++	0	0
From throat[1] in rubella*														
From throat in rubella....	0								+	+	+	±	0	
From throat in rubella....	++	0							0					0
From blood in rubella.....	++	0							0					0
From blood in rubella.....	++	0							0					0
Epidemic poliomyelitis....	0								+	+	0	0		0
Acute bronchitis..........	+	+	0						0					0

* Agglutinated spontaneously.
1. Strains used to immunize rabbits.
++ Marked clumping with complete clearing of the mixtures.

OPSONINS

The serum was diluted with normal salt solution, and then equal parts of washed human leukocytes and bacteria were added, making a total dilution of 1:15. At this dilution most of the normal opsonins were removed. The specimens were incubated at 37 C. for 15 minutes and the smears then stained with carbolthionin. Fifty polymorphonuclear leukocytes were counted and the number of cells taking part in phagocytosis noted. When the difference in phagocytic power between the immune and control serum was not marked, the experiments were repeated to be certain that the slight differences were of significance.

The serums were diluted to the point of opsonin extinction from time to time, the results corresponding then to the results obtained by diluting the

TABLE 2.

Opsonin Determinations

Sources of Cocci	Normal Rabbit Serum	Serum of Rabbit Immunized With Coccus from Measles	Serum of Rabbit Immunized With Coccus from Rubella
From measles: Blood	8	32	7
Blood	6	22	12
Eye	6	24	6
Ear	3	14	6
Nose	2	12	2
Throat	0	16	6
Throat	2	22	2
Throat	6	30	2
From rubella: Blood	4	8	30
Blood	0	2	26
Blood	7	9	36
Blood	2	2	16
Blood	0	2	7
Blood	4	6	28
Throat	8	8	32
Throat	7	11	22
Throat	6	6	42
Throat	0	2	30
Throat	0	2	14
Throat	3	2	13
Throat	0	0	20
Throat	4	2	28
Throat	0	0	9
Throat	3	3	13
Throat	2	2	7
Throat	6	7	16
Throat	4	2	10
Throat	4	8	16
Throat	6	8	8
Throat	1	0	4
Throat	2	8	20
Throat	5	7	16
Throat	0	1	9
Other sources:			
Arthritis	6	0	0
Endocarditis, blood	8	6	2
Tonsillitis	10	8	24
Tonsillitis	4	4	4
Tonsillitis	14	14	12
Tonsillitis	18	12	18
Tonsillitis	0	2	0
Acute chorea, throat	4	7	13
Acute chorea, throat	2	4	4
Pyorrhea	0	0	0
Pyorrhea	4	15	3
Spleen	8	6	8
Horse streptococcus	3	1	2
Poliomyelitis	5	4	7
Poliomyelitis	10	10	11
Poliomyelitis	3	3	1
Poliomyelitis	1	1	1
Poliomyelitis	0	1	0
Poliomyelitis	0	0	0
Pneumococcus, Type I	4	2	0
Pneumococcus, Type I	2	0	2
Pneumococcus, Type II	15	12	10
Pneumococcus, Type II	6	4	6
Pneumococcus, Type III	2	0	2
Pneumococcus, Type IV	10	6	8
Guinea-pig, blood	0	2	4
Streptococcus hemol.			
Tonsillitis	2	2	2
Scarlet fever, throat	6	10	9
Scarlet fever, blood	6	6	6
Scarlet fever, gland	20	20	20

serums to 1 : 15. The highest point of opsonin extinction by the measles serum was 1 : 960 for a heterologous strain. The highest point reached by the rubella strains was 1 : 4,800 for an homologous organism. The point of opsonin extinction for the control serum was 1 : 30.

The results (Table 2) show that the serum of an immune measles rabbit has marked opsonic power for measles diplococci, while the serum of an immune rubella rabbit and a control rabbit have about the same low opsonic content for these organisms. Further, that the serum of an immune rubella rabbit has increased opsonic power for rubella diplococci, while the measles and control rabbits have no such power.

The immune measles and rubella rabbit serum did not show any increase in opsonins for the 32 strains of diplococci and streptococci isolated from other sources, except in 3 instances, 1 a diplococcus from the throat of a nurse of a rubella patient, 1 from the throat of an acute chorea case, both of which corresponded morphologically to the rubella coccus and responded to the rubella rabbit serum, and small round diplococcus producing green colonies on blood agar isolated from a case of pyorrhea, reacted to the measles rabbit serum.

The opsonic determinations appear to show quite specific reactions which may be a help in differentiating the measles and rubella diplococci from each other and from similar organisms.

SUMMARY

Complement fixing bodies for the measles and rubella diplococci are present in the blood of measles and rubella patients to a slight extent as the symptoms subside.

The blood of rabbits injected with various strains of measles and rubella diplococci develop agglutinins and opsonins.

Agglutinins are demonstrable in the blood of immune measles rabbits often in high dilutions, both with homologous and heterologous measles strains. Satisfactory agglutination experiments are not possible as a rule until after long isolation of the diplococcus on account of spontaneous agglutination.

Agglutinins are present in the serum of immune rubella rabbits, but generally only for the homologous strains.

Specific opsonins are demonstrable in the blood of immune measles and rubella rabbits in high dilutions, and furnish the easiest and most reliable immunological method so far found, to differentiate measles and rubella diplococci from each other and from other nonhemolytic diplococci and streptococci.

A PLATE METHOD FOR ISOLATING ANAEROBES

George F. Dick

From the Laboratory of St. Luke's Hospital, Chicago.

Methods previously described for plating anaerobes have involved the use of an anaerobic chamber or the inversion of the bottom of the Petri dish in the top and sealing with paraffin. The anaerobic chamber is not always available and is cumbersome. The inverted plate method is simple, but the plate, once opened, cannot be sealed again, and in opening, the agar frequently breaks, part clinging to the bottom and part to the top of the Petri dish. The layer of agar is also apt to slide over the glass so that there is danger of originally well isolated colonies becoming mixed. In the following method these difficulties are avoided.

Dilutions of the material to be plated are made in the usual way; melted deep agar tubes are inoculated and plates poured. The plates are immediately chilled and as soon as the agar sets, another deep tube of sterile agar is poured over the surface of each plate. The plates are again chilled to set the second layer of agar, and the surface of each is covered with a layer of melted paraffin about 2-3 mm. thick, care being taken to have the paraffin completely cover the surface of the agar. The second tube of agar should be cooled to 42 C. before it is poured over the surface of the plate. If it is used too hot it may kill the organisms in the first layer of agar. The paraffin is sterilized by heating to the smoking point in a shallow tin or aluminum cup. It is cooled until a film can be seen forming on the surface and then rapidly spread over the plates by a warm sterile spoon. Three or four ordinary teaspoonfuls cover the surface of a 100 mm. dish. The spoon is sterilized by flaming or by heating in the paraffin.

In order to open the plates, a thin section lifter is sterilized in the flame, cooled and used to lift the whole layer of paraffin; or blocks of paraffin may be cut out over the colonies to be examined without disturbing the rest of the plate. After the colonies are removed, the breaks in the paraffin covering may be sealed with more melted paraffin and the plates incubated longer. It is best to remove the colonies with sterile glass pipets drawn out to points that are not too fine. With care the top layer of agar may be lifted from the bottom layer, but this is usually not necessary.

Tetanus and gas bacilli grow well by this method. The tetanus bacillus colonies appear at the end of 24 hours' growth in dextrose agar as minute opaque points. At the end of 48 hours, they have grown into fleecy white, more or less spherical colonies about 2.5 mm. in diameter. If the plates are too thickly inoculated, the colonies of tetanus bacillus do not attain such size or acquire so much of the fleecy appearance.

Gas bacilli grown on blood-agar plates appear as small grayish colonies with a wide zone of hemolysis.

This method has been in use as a routine for 18 months, and there has been no difficulty in avoiding contaminations.

GENERAL INDEX

A

B

C

E

F

G

H

M

N

P

S

T

RC
110
J67
v.23
cop.4

The Journal of infectious
diseases

Biological
& Medical
Serials

PLEASE DO NOT REMOVE
CARDS OR SLIPS FROM THIS POCKET

UNIVERSITY OF TORONTO LIBRARY